Transport of β-Lactam Antibiotics in Kidney Brush Border Membrane

Determinants of Their Affinity for the Oligopeptide/H^+ Symporter

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Abstract

This study was designed to determine whether β-lactam antibiotics (cephalosporins and penicillins) are all substrates for the renal oligopeptide/H^+ symporter and, if so, whether the transport system discriminates among the numerous β-lactam antibiotics. We used [^{3}H]glycylglutamine, [^{3}H]cephalexin, and [^{3}H]-ampicillin as probes for the transport of oligopeptides, cephalosporins, and penicillins in kidney brush border membrane vesicles, respectively. Among the β-lactam antibiotics, only those with an α-amino group in the phenylacetamido moiety were found to interact with the oligopeptide/H^+ symporter. Aminocephalosporins displayed high affinities (K_is generally < 250 μM), whereas aminopenicillins displayed low affinities (K_i 0.78–3.03 mM). These differences in affinities appeared to be a consequence of conformational features of the substrates, especially the sterical location of the carboxy group. The affinities of aminolactams for the oligopeptide/H^+ symporter were, furthermore, related to the hydrophobicity of the phenylglycyl chains and the substituents attached to the thiazolidine and dihydrothiazine ring. In sharp contrast to the uptake of [^{3}H]-glycylglutamine and [^{3}H]cephalexin, the uptake of [^{3}H]-ampicillin was not dependent on a pH gradient and was inhibited by various β-lactam antibiotics, whether or not they contained an α-amino group. Our data suggest that: (*a*) the transport of aminocephalosporins is largely mediated by the oligopeptide/H^+ symporter, which is highly influenced by the substrate structure; and (*b*) penicillins are transported by another system, which is less discriminative with respect to substrate structure. (*J. Clin. Invest.* 1993. 92:2215–2223.) Key words: peptides • antibiotics • transport • kidney • brush border membrane

Introduction

β-Lactam antibiotics (cephalosporins and penicillins) are widely used for the treatment of bacterial infections. The kidney has a key role in the elimination of these antibiotics from plasma, since they are almost completely excreted into the urine whether administered orally or intravenously (1, 2). The urinary excretion appears to be the result of two processes: glomerular filtration and secretion into the tubular lumen (1, 3). Although the transepithelial flux of β-lactam antibiotics has not been studied in detail, the following steps seem to be involved in the latter process. There is evidence that the β-lactam antibiotics, circulating in the blood, are taken up into tubular cells by means of the paraaminohippuric acid transport system located in the basolateral membrane (4). Efflux of the antibiotics from the tubular epithelium into the lumen is probably mediated by multiple transport systems, including anion and cation exchangers located in the brush border membrane (5–7).

A recent study has suggested that the renal handling of β-lactam antibiotics may also include tubular reabsorption. This suggestion is based on the finding (8) of a transport system for β-lactam antibiotics in kidney brush border membrane vesicles (BBMV).[1] Even more striking is that apparently β-lactam antibiotics and oligopeptides share the same transport system.

Although a common transport system for two seemingly dissimilar groups of substrates may appear unusual, an examination of the structures of these compounds shows that the basic configuration of β-lactam antibiotics resembles the backbone of a tripeptide, with the COOH-terminal peptide bond incorporated into a four-membered β-lactam ring (Fig. 1). This ring is fused into either a six-membered dihydrothiazine ring, in the case of cephalosporins, or a five-membered thiazolidine ring, in the case of penicillins (9). Variations in the configuration of β-lactam antibiotics occur predominantly at R_1 of both penicillins and cephalosporins (Fig. 1) as well as at R_3 in the dihydrothiazine ring of cephalosporins (Fig. 1). The amino group of aminopenicillins and aminocephalosporins is attached at R_2.

Studies from several laboratories, including our own (10–12), have shown that the uptake of oligopeptides across the brush border membrane of kidney tubular cells is mediated by two distinct transport systems. One is characterized as the high-affinity/low-capacity and the other as the low-affinity/high-capacity oligopeptide transporter. In addition, we found that the high-affinity system does not operate in the absence of a pH gradient, whereas the low-affinity transport system does (11).

The objectives of the present experiment, using kidney BBMV, included the investigation of the following questions. (*a*) Is either the oligopeptide/H^+ symporter or the low-affinity carrier system involved in the transport of β-lactam antibiotics? (*b*) Does the transport system discriminate among the numerous β-lactam antibiotics? The apparent affinities of β-lactam antibiotics for the oligopeptide/H^+ symporter were determined by the inhibition of [^{3}H]glycylglutamine uptake into kidney BBMV. In addition, we used [^{3}H]cephalexin and [^{3}H]-ampicillin as model compounds representing cephalosporins and penicillins, respectively.

Methods

Adult male Sprague-Dawley rats with a body weight of 238±10 g were purchased from Zivic-Miller (Allison Park, PA). Custom-synthesized

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1. *Abbreviations used in this paper:* BBMV, brush border membrane vesicles; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

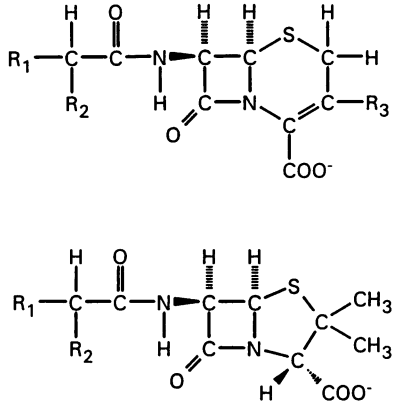

Figure 1. General molecular structures of cephalosporins (*top*) and penicillins (*bottom*).

[glutamine-3,4-^{3}H]glycylglutamine (49 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [^{3}H]Ampicillin (41.6 mCi/mmol) was provided by SmithKline & Beecham (Betchworth, Great Britain) and [^{3}H]cephalexin (3.6 Ci/mmol) was a gift from SmithKline & Beecham (King of Prussia, PA). The β-lactam antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO) or provided by Eli Lilly and Co. (Indianapolis, IN). The oligopeptides were purchased from Bachem Bioscience Inc. (Philadelphia, PA). Reagents for protein-dye binding were obtained from Bio-Rad Laboratories (Richmond, CA).

Preparation of BBMV. BBMV were prepared from the cortex and outer medulla of rat kidneys using a Mg^{2+}/EGTA precipitation technique as previously described (11). Determination of the specific activity of the membrane marker enzymes in the final membrane preparation in relation to the starting homogenate revealed no enrichment of K^+-stimulated ouabain-sensitive Na^+/K^+-ATPase, but a 13-fold enrichment for alkaline phosphatase and 16-fold enrichment for γ-glutamyl transferase. BBMV were loaded, if not stated otherwise, with 50 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4 (pH 8.3).

Uptake of [^{3}H]glycylglutamine, [^{3}H]cephalexin, and [^{3}H]ampicillin. Uptake studies were performed at 24°C by using a rapid filtration technique with filters (HAWP type, 0.45-μm pore size; Millipore Corp., Bedford, MA) as described previously (11). Uptake was initiated by rapidly mixing 20 μl of membrane suspension (150 μg of protein) preloaded with buffer (pH 8.3) with 80 μl of medium consisting of 50 mM Hepes, 50 mM 2-[*N*-morpholino]ethanesulfonic acid (Mes), 25 mM Tris, and 300 mM mannitol (pH 6.0) and 4.9 μCi/ml of [^{3}H]glycylglutamine or 4.26 μCi/ml of [^{3}H]ampicillin. The resulting final pH was 6.7. Incubation of vesicles was terminated by a 50-fold dilution with an ice-cold stop solution (2 mM Hepes/Tris, 210 mM KCl, pH 7.5), followed by filtration. The filters were washed with 4 ml of stop solution. The radioactivity associated with the filter was counted in 10 ml HiSafe III (Pharmacia LKB, Piscataway, NJ) in a β-scintillation counter (Packard Tricarb, Downers Grove, IL). Unspecific binding of the labeled compounds to the filter (< 1% of uptake) was subtracted from the transport data.

The experiments determining the structure–affinity relationship of the β-lactam antibiotics were performed with 5 s of incubation at a [^{3}H]glycylglutamine concentration of 0.1 μM in the presence of an inwardly directed pH gradient of 1.6 units. This pH gradient maximally stimulated dipeptide uptake into BBMV (11, 13). All competitors were added to the incubation medium in concentrations between 0.1 μM and 10 mM. Medium osmolarity was adjusted to 440 mosmol/liter by the addition of mannitol.

Uptake of [^{3}H]cephalexin into kidney BBMV was determined as a function of incubation time in the presence and the absence of a transmembrane pH gradient as described above with a cephalexin concentration of 0.5 μM. To distinguish between transport into an osmotically reactive space and binding to the membrane, pH gradient–dependent [^{3}H]cephalexin uptake (1.65 μM) was studied in the presence of increasing concentrations of mannitol. Uptake was terminated by ice-cold stop buffer (pH 7.2) adjusted to the same osmolarity as the incubation medium. Kinetics of [^{3}H]cephalexin uptake as a function of cephalexin concentration was determined at 5 s of incubation in a concentration range of 5 μM to 20 mM. BBMV were preloaded as described above and incubated in a buffer of pH 6.0 (pH_{in} 8.3/pH_{out} final 6.7). To compare the potencies of a variety of β-lactam antibiotics and oligopeptides for inhibition of [^{3}H]glycylglutamine and [^{3}H]-cephalexin uptake, BBMV were incubated for 5 s with 0.1 μM [^{3}H]-glycylglutamine or 1 μM [^{3}H]cephalexin in the absence or presence of 1 mM of the following compounds: glycylglutamine, cephalexin, amoxicillin, cefadroxil, cephaloglycin, benzylpenicillin, and Gly-Gly-Gly.

Influx of ampicillin as a function of time in the presence and absence of a transmembrane pH gradient was investigated in vesicles preloaded as described above and incubated in a buffer of pH 6.0 or 8.3 containing 100 μM [^{3}H]ampicillin. To compare ampicillin uptake with the uptake of glycylglutamine, the same experiments were performed using 100 μM [^{3}H]glycylglutamine. When uptake of ampicillin was studied as a function of ampicillin concentration, both in the presence and the absence of inhibitors, BBMV were preloaded with a buffer of pH 7.4 (45 mM Hepes, 40 mM Mes, 40 mM Tris, and 300 mM mannitol). Incubation was carried out as described above in buffer pH 7.4 containing 100 μM to 25 mM ampicillin, 5 μCi/ml [^{3}H]ampicillin, and 20 mM either glycylglutamine, cephalexin, or benzylpenicillin.

Metabolism of β-lactam antibiotics. To determine whether [^{3}H]-cephalexin and [^{3}H]amoxicillin are metabolized in the incubation medium or within the BBMV during the course of the uptake experiments, HPLC-analysis was used. BBMV were incubated for 30 min at 25°C in the presence of 0.1 mM [^{3}H]cephalexin or 0.1 mM [^{3}H]-amoxicillin at pH 6.7. After termination of incubation, samples were processed as described previously (11) and chromatography was performed on a reversed-phase C18 column (Bondapak, Milford, MA). Solvent A was 0.1% trifluoracetic acid (TFA) in H_2O and solvent B was 95% CH_3CN, 0.1% TFA, 5% H_2O. A linear gradient from 100% A to 100% B was run over a 15-min period. Compounds were detected at 278 nm, the eluent was fraction-collected, and radioactivity was measured in 1-ml samples of the eluent. Identity of the peaks was established by cochromatography with the original radiolabeled compounds.

Calculations and statistics. We used apparent K_i values as a measure of affinity, assuming competitive interaction of substrate and inhibitor at the binding site on the transporter. The calculation of K_i values was based on the EC_{50} values (effector concentration causing 50% inhibition of uptake of the tracer) as derived by a nonlinear regression analysis of the competition curves with one component using INPLOT (GraphPAD, Los Angeles, CA). The use of a very low substrate concentration simplifies the determination of K_m and K_i since, in this case, the $[S] + K_m$ term is reduced to K_m without serious error and K_i equals the concentration that reduces the rate of transport by one-half (14, 15). In these experiments, the substrate concentration was adjusted to 0.1 μM and was, therefore, 500 times lower than the K_m or K_i of glycylglutamine, 56.2±8.0 and 46.2±1.1 μM, respectively (11, 13).

To derive the kinetic constants for pH gradient–dependent uptake of [^{3}H]cephalexin into BBMV as a function of [*S*], velocity data were fitted by iteration to an equation involving the sum of two saturable components plus a linear term. Analysis was performed by using INPLOT with initial estimates as derived from inspection of the Eadie-Hofstee plots.

All experiments were carried out in triplicate with at least three preparations; results are presented as the mean±SE. All kinetic constants were determined by linear regression analysis by the least-squares method. Statistical comparisons of the slopes and kinetic constants were performed by ANOVA followed by a Bonferroni-corrected *t* test. Significance of differences between the inhibitors was determined by a nonpaired *t* test.

It is pertinent to mention a limitation of our study. On the basis of the competition experiments, we cannot conclude, a priori, that all

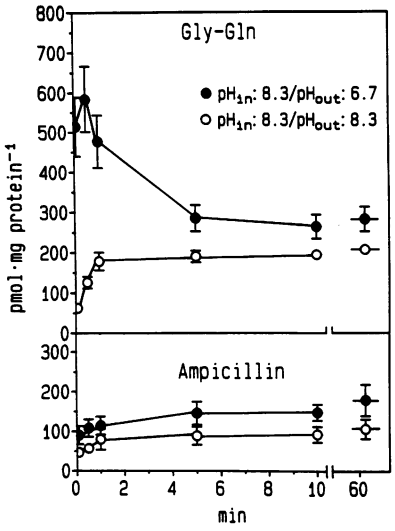

Figure 2. Uptake of glycylglutamine and ampicillin as a function of time in absence and presence of a transmembrane pH gradient. BBMV were preloaded with buffer pH 8.3 (45 mM Hepes, 75 mM Tris, 100 mM K_2SO_4). After preloading, BBMV were incubated in a buffer with a pH of either 6.0 (*filled circles*) or 8.3 (*open circles*). The acidic buffer contained 50 mM Hepes, 50 mM Mes, 20 mM Tris, and 300 mM mannitol (final pH = 6.7). The basic buffer contained 45 mM Hepes, 75 mM Tris, and 300 mannitol. Each buffer contained either 0.1 mM [^{3}H]glycylglutamine or 0.1 mM [^{3}H]ampicillin. Uptake rates represent mean±SE of three membrane preparations.

compounds showing a high affinity for the interaction with the oligopeptide/H^+ symporter are indeed transported across the brush border membrane. The apparent K_i values allow only the description of the affinity for interaction at the substrate binding site of the transporter.

Results

The following studies were performed with [^{3}H]glycylglutamine serving as the probe for characterizing the oligopeptide/H^+ symporter and the interaction of β-lactam antibiotics with the substrate binding site of the transporter.

Uptake as a function of time. Initially, we determined the time course of uptake of 100 μM [^{3}H]glycylglutamine into BBMV in the absence and presence of a transmembrane pH gradient (pH_{in} 8.3, pH_{out} 6.7). A pronounced overshoot phenomenon was observed in the presence of a pH gradient, but not in the absence of a pH gradient (Fig. 2). At 20 s of incubation, the uptake in the presence of a pH gradient was about fourfold higher than in the absence of a pH gradient (Fig. 2). We have previously shown that the initial glycylglutamine influx (up to 30 s) into renal BBMV represents entirely uptake of the intact dipeptide (11). Furthermore, glycylglutamine is taken up into an osmotically reactive space with negligible binding to the membrane (11). Since the influx of glycylglutamine during the initial 8 s was linear, we used an incubation time of 5 s for all the subsequent studies.

Type of interaction. The interaction of β-lactam antibiotics with [^{3}H]glycylglutamine at the substrate binding site could be either competitive, noncompetitive, uncompetitive, or partially competitive. To determine the type of interaction, uptake of 0.2, 0.3, and 1.0 μM [^{3}H]glycylglutamine was measured in the presence of increasing concentrations of cephalexin, loracarbef, and amoxicillin (0.1–1 mM). Uptake rates of glycylglutamine were plotted as a function of the inhibitor concentration according to Dixon, which allows one to distinguish between competitive and noncompetitive on the one hand and uncompetitive and partially competitive inhibition on the other. All compounds exhibited kinetics (Fig. 3) that were consistent with either competitive or noncompetitive inhibition. Because noncompetitive inhibition is quite uncommon, we have assumed that the interaction of the compounds and glycylglutamine at the carrier site is of a competitive nature. The apparent K_i values determined by regression analysis from the Dixon plot were 63.1±1.1 μM for cephalexin, 96.8±4.1 μM for loracarbef, and 492.8±79.3 μM for amoxicillin.

Relation between structure of cephalosporins and affinity. Cephalosporins vary in structure because of the different configurations of R_1, R_2, and R_3 and depending on whether carbon or sulfur occupies the first position of the dihydrothiazine ring (Fig. 1). The purpose of the following experiments was to investigate the influence of these configurational variations on the affinity of cephalosporins for binding to the oligopeptide/H^+ symporter.

Cephalosporins have either NH_2 or H as the R_2 substituent. All of the various α-amino cephalosporins examined showed a rather high affinity for the binding site of the transporter (Table I). The K_i values ranged between 55.9±3.6 (cefadroxil) and 1,561±482 μM (cephaloglycin). In contrast, cephalosporins without an α-amino group, regardless of their particular configuration, such as those shown in Table I, did not interact with

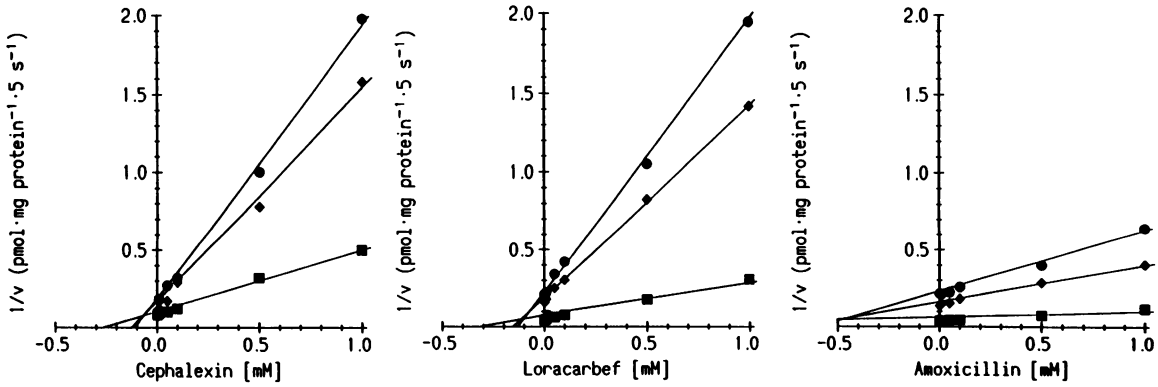

Figure 3. Dixon plot (1/*v* over *I*) of glycylglutamine uptake in presence of increasing concentrations of cephalexin, loracarbef, and amoxicillin. BBMV were preloaded with a buffer with a pH of 8.3 (45 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4). After preloading, BBMV were incubated for 5 s in a buffer with a pH of 6.0 containing 50 mM Hepes, 50 mM Mes, 20 mM Tris, 300 mM mannitol, and increasing concentrations of [^{3}H]glycylglutamine and cephalexin (final pH = 6.7). Uptake rates represent mean±SE of three membrane preparations.

Table I. Configuration and Affinity of Cephalosporins

Pos. 1	R_1	R_2	R_3	Name	K_i
					μM
S	C_6H_5	NH_2	Cl	Cefaclor	60.1±3.2
S	C_6H_5	NH_2	CH_3	Cephalexin	63.1±1.1
S	C_6H_7	NH_2	CH_3	Cephradine	124.2±5.9
S	C_6H_7	NH_2	OCH_3	Cefroxadine	210.6±18.2
S	C_6H_4OH	NH_2	CH_3	Cefadroxil	55.9±3.6
S	C_6H_5	NH_2	CH_2OCOCH_3	Cephaloglycin	1,561.0±482.0
C	C_6H_5	NH_2	Cl	Loracarbef	84.1±1.1
S	SC_5H_4N	H	CH_2OCOCH_3	Cefapirin	>10,000
S	SC_5H_4	H	$CH_2NC_5H_4$	Cephaloridine	>10,000
S	SC_5H_4	H	CH_2OCOCH_3	Cephalothin	>10,000
S	$(CH_2)_2CHNH_2COOH$	H	CH_2OCOCH_3	Cephamycin C	>10,000
S	OC_6H_5	H	H	LY289790*	>10,000

Apparent K_i values were derived from inhibition plots of the uptake of [^{3}H]glycylglutamine incubated in the presence of increasing concentrations of cephalosporins. BBMV were preloaded with a buffer of pH 8.3 (45 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4) and incubated for 5 s in buffer pH 6.0 (50 mM Hepes, 50 mM Mes, 20 mM Tris, and 300 mM mannitol) in presence of 0.1 μM to 10 mM of the cephalosporins. K_i values represent mean ± SE of three membrane preparations. * Blocked carboxy group.

the transporter, as judged by apparent K_i values of, generally, > 10 mM.

To investigate the impact of the various R_3 substituents on affinity, we determined the apparent K_i values of pairs of cephalosporins that differed only with respect to the configuration of R_3 (Table I); for example, cefaclor and cephalexin (Cl vs. CH_3), cephradine and cefroxadine (CH_3 vs. OCH_3), and cephalexin and cephaloglycin (CH_3 vs. CH_2OCOCH_3). Cephalosporins with a hydrophobic substituent at R_3 like CH_3 (lipophilic constant = 0.71 [16]) had a significantly higher affinity ($P < 0.01$) than cephalosporins with more polar substituents like OCH_3 (lipophilic constant = −0.02) or CH_2OCOCH_3 (lipophilic constant = −0.17). There was no significant difference between the K_i values of cefaclor and cephalexin. This corresponds to a lack of difference between lipophilic constants (0.71 vs. 0.56) of their R_3 substituents (Cl vs. CH_3). These studies show that a more lipophilic residue attached at R_3 confers a higher affinity for the carrier.

The influence of R_1 substitutions on affinity was investigated by determining the K_i values of four aminocephalosporins that differed in this respect. Cephradine has a more saturated and, therefore, more polar ring system than cephalexin. This structural feature caused a significant ($P < 0.01$) increase in K_i value (63.1±1.2 vs. 124±5.9 μM). On the other hand, introducing a hydrophilic OH group (lipophilic constant = −0.67) into the phenyl group of cephalexin to yield cefadroxil failed to cause a reduction in affinity (63.1±1.1 vs. 55.9±3.6 μM).

The importance of the conformation of α-aminocephalosporins was studied by determining the K_i values for loracarbef and epiloracarbef. Loracarbef is homologous to cefaclor, except that the sulfur atom of the dihydrothiazine ring is substituted by a carbon atom. This exchange reduced the apparent affinity (app. K_i) from 60.1±3.2 μM (cefaclor) to 84.1±1.1 μM (loracarbef), indicating that sulfur in the dihydrothiazine ring is not of great importance in overall substrate recognition. In contrast to this structural difference, conformation seemed to be critical for a high affinity interaction with the binding site. As shown in Fig. 4, in the case of epiloracarbef, much higher concentrations were necessary to cause 50% inhibition of glycylglutamine uptake than in the case of loracarbef. The apparent affinity of epiloracarbef was seven times lower (K_i 602.1±3.2 μM) than that of loracarbef.

The conformation of cephalosporins in relation to affinity was further elucidated in regard to the stereospecificity of interaction with the carrier site. We determined the K_i values for D- and L-isomers of two aminocephalosporins, cephalexin and loracarbef. The apparent affinity of L-cephalexin was significantly ($P < 0.01$) higher than that of its D-isomer (K_i 43.7±1.2 vs. 63.1±1.1 μM) and that of L-loracarbef was more than four times higher than that of its D-isomer (K_i 19.7±2.0 vs. 84.1±1.1 μM). These comparisons indicate a stereospecific interaction of the compounds with the substrate binding site.

The results of the above studies suggested that the transport of aminocephalosporins into renal BBMV is mediated by the oligopeptide/H^+ symporter. To verify this suggestion, the following studies were performed with [^{3}H]cephalexin serving as the probe.

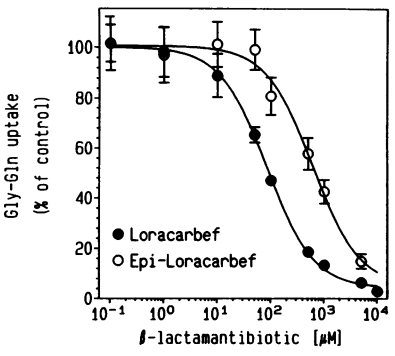

Figure 4. Uptake of glycylglutamine, as percentage of control, in presence of increasing concentrations of loracarbef or epiloracarbef. BBMV were preloaded with a buffer with a pH 8.3 (45 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4). After preloading, BBMV were incubated for 5 s in a buffer with a pH of 6.0 containing 50 mM Hepes, 50 mM Mes, 20 mM Tris, 300 mM mannitol, 0.1 μM [^{3}H]glycylglutamine, and increasing concentrations (0.1 μM to 10 mM) of β-lactam antibiotics. Uptake rates represent mean±SE of three membrane preparations.

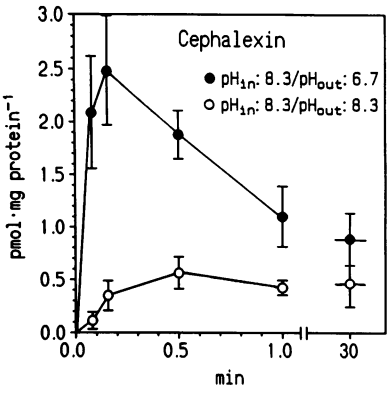

Figure 5. Uptake of cephalexin as a function of time in absence and presence of a transmembrane pH gradient. BBMV were preloaded with buffer pH 8.3 (45 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4). After preloading, BBMV were incubated in a buffer with a pH of either 6.0 (*filled circles*) or 8.3 (*open circles*). The acidic buffer contained 50 mM Hepes, 50 mM Mes, 20 mM Tris, and 300 mM mannitol (final pH = 6.7). The basic buffer contained 45 mM Hepes, 75 mM Tris, and 300 mannitol. Each buffer contained 0.5 μM [^{3}H]-cephalexin. Uptake rates represent mean±SE of three membrane preparations.

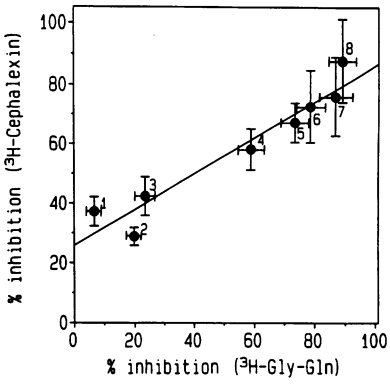

Figure 7. Correlation between the potencies of various β-lactams and peptides to inhibit [^{3}H]glycylglutamine and [^{3}H]cephalexin uptake. Uptake (5 s) was measured with 0.1 μM [^{3}H]glycylglutamine or 1 μM [^{3}H]cephalexin in the presence of a transmembrane pH gradient and 1 mM of the following compounds: (*1*) benzylpenicillin, (*2*) ampicillin, (*3*) cephaloglycin, (*4*) amoxicillin, (*5*) Gly-Gly-Gly, (*6*) cephalexin, (*7*) glycylglutamine, and (*8*) cefadroxil. Uptake rates represent mean±SE of three membrane preparations. Least-square regression: y-intercept = 26.73±4.74, slope = 0.611±0.075, r^2 = 0.9158, P = 0.0002.

Cephalexin uptake. Uptake of [^{3}H]cephalexin into kidney BBMV was determined at various time intervals in the presence and the absence of an inwardly directed pH gradient (Fig. 5). In the presence of a pH gradient, a pronounced overshoot of cephalexin uptake was observed at 10 s, which exceeded the equilibrium value by fourfold. An overshoot did not occur in the absence of a pH gradient. Furthermore, the results of the preliminary studies showed that this uptake, like that of glycylglutamine (11), was predominantly transport into an osmotically reactive space. There was no metabolism of [^{3}H]-cephalexin in BBMV as judged by recovery of the intact compound by HPLC analysis.

When uptake of [^{3}H]cephalexin was determined in the presence of a pH gradient as a function of cephalexin concentration (5 μM to 20 mM), saturation kinetics was observed (Fig. 6). The Eadie-Hofstee plot of the data shown in the inset to Fig. 6 after subtraction of a diffusional component reveals the presence of a high-affinity and a low-affinity transport system. The kinetic constants of the two affinity sites were determined by nonlinear regression analysis and least-square fitting. The high-affinity site has an apparent K_m of 80.3±38.6 μM and a V_{max} of 0.51±0.16 nmol · mg protein^{-1} · 5 s^{-1}. The low-affinity site displays an apparent K_m of 8.2±2.0 mM and a V_{max} of 13.97±2.71 nmol · mg protein^{-1} · 5 s^{-1}. The diffusional component was determined to be 0.12±0.03 nmol · mg protein^{-1} · 5 s^{-1} · mM^{-1}. These results indicate that the two transporters differ by 100-fold with respect to substrate affinity and by 25-fold with respect to maximal velocity.

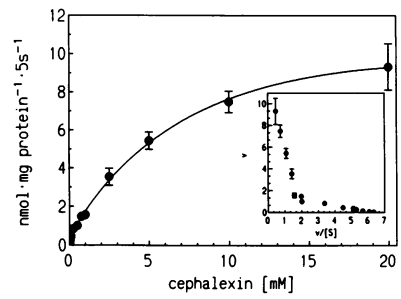

Figure 6. Kinetics of [^{3}H]cephalexin uptake as a function of cephalexin concentration in the presence of a pH gradient. Membrane vesicles were preloaded with buffer pH 8.3 (45 mM Hepes and 75 mM Tris) containing 100 mM K_2SO_4 and incubated for 5 s in buffer containing 50 mM Hepes; 20 mM Tris, pH 6.0 (final pH 6.7); and 280–300 mM mannitol according to the concentration of cephalexin (5 μM–20 mM) to equalize medium osmolarity. Uptake data represent the saturable component after subtraction of a minor diffusional component. (*Inset*) Eadie-Hofstee plot (v vs. $v/[S]$) of saturable cephalexin uptake, where v is the rate of uptake and [S] the cephalexin concentration in millimolar. The data represent mean±SE of three membrane preparations.

Inhibition of cephalexin uptake. We first determined the effect of adding glycylglutamine (1 mM) on the uptake of [^{3}H]-cephalexin (1 μM) by the renal BBMV. There was 76±13% inhibition of cephalexin uptake. We then investigated to what extent a variety of β-lactams and peptides inhibit uptake of [^{3}H]glycylglutamine and [^{3}H]cephalexin under the same experimental conditions. The only difference was that the concentration of Gly-Gln was 0.1 μM, whereas that of cephalexin was 1 μM. This difference in concentration was necessitated by the lower specific activity of [^{3}H]cephalexin than [^{3}H]Gly-Gln. As shown in Fig. 7, there was a highly significant correlation (r^2 = 0.9158, P = 0.0002) in the potencies of the various antibiotics and peptides to inhibit the uptake of both probes.

Relation between the structure of penicillins and affinity. In general, penicillins (Table II) caused much less inhibition of [^{3}H]glycylglutamine uptake than did the aminocephalospo-

Table II. Configuration and Affinity of Penicillins

R_1	R_2	Name	Apparent K_i
			mM
C_6H_5	NH_2	Amoxicillin	0.78±0.05
C_6H_4OH	NH_2	Ampicillin	3.03±0.23
C_6H_7	NH_2	Epicillin	2.07±0.17
C_6H_5	H	Benzylpenicillin	>10
C_6H_5	COOH	Carbenicillin	>10
C_6H_5	NCH_2	Metampicillin	2.62±0.51

Apparent K_i values were derived from inhibition plots of the uptake of [^{3}H]glycylglutamine in presence of increasing concentrations of penicillins. The preloading and incubation conditions of BBMV were the same as those for cephalosporins (see legend to Table I). K_i values represent mean±SE of three membrane preparations.

rins (Table I). The K_i values for the aminopenicillins ranged between 0.78±0.05 mM for amoxicillin and 3.03±0.23 mM for ampicillin. As in the case of cephalosporins, the presence of an α-amino group appeared to be critical. The substitution of the α-amino group of amoxicillin by a H atom, as in benzylpenicillin, or by a carboxy group, as in carbenicillin, abolished the interaction with the substrate binding site of the transporter. However, there was still some measurable affinity when the α-amino group was substituted by a methylene amino group, as in metampicillin (Table II).

Last, to study the impact of various configurations of R_1 in aminopenicillins, we compared the K_i values of amoxicillin, ampicillin, and epicillin (Table II). The more polar hydroxyphenyl ring in ampicillin, as well as the more saturated (hydrogenated) phenyl ring in epicillin, caused a significant reduction in apparent affinity by three to four times compared with amoxicillin. This demonstrates that a more hydrophobic domain in the NH_2-terminal side chain of aminopenicillins increases the affinity for interaction with the binding site of the oligopeptide/H^+ symporter.

The above studies indicated that aminopenicillins generally have a poor affinity for the oligopeptide/H^+ symporter. To investigate whether these compounds might use another transport system, the following studies on the uptake of [^{3}H]-ampicillin into renal BBMV were performed.

Time course of ampicillin uptake. In contrast to [^{3}H]-glycylglutamine, uptake of 100 μM [^{3}H]ampicillin never exceeded its equilibrium value and was not affected by imposing an inwardly directed H^+ gradient (Fig. 2). Equilibrium uptake of ampicillin in the presence of 820 mM mannitol in the medium (final osmolality = 950 mosmol/liter) was reduced to 40±8% of the uptake rate at 300 mM mannitol, indicating transport of ampicillin into an osmotically reactive space with a minor membrane binding component. Determination of ampicillin influx in the presence and absence of inwardly directed transmembrane Na^+ or K^+ gradients did not reveal any stimulation by a Na^+ gradient, nor was an overshoot phenomenon observed (data not shown). The HPLC analysis showed there was no metabolism of [^{3}H]ampicillin in BBMV.

Inhibition of ampicillin uptake. Transport of ampicillin (0.35 mM) into renal BBMV was measured in the absence and the presence of 20 mM glycylglutamine or various β-lactam antibiotics. These experiments were performed in the absence of a transmembrane pH gradient ($pH_{in} = pH_{out} = 7.4$) because the results of the studies on the time course of ampicillin uptake indicated that a transmembrane pH gradient is not necessary as a driving force.

Glycylglutamine, as well as various cephalosporins and penicillins, inhibited ampicillin uptake significantly ($P < 0.05$) by 30–55% (Table III). However, the specificity of interaction was not similar to that observed with glycylglutamine serving as the probe under the conditions of a transmembrane pH gradient. For example, cephalosporins and penicillins, with or without an α-amino group, inhibited uptake to almost the same extent.

Interaction of ampicillin and competitors. For this experiment, we determined the kinetics of [^{3}H]ampicillin uptake as a function of ampicillin concentration (0.15–25 mM) in the absence and presence of 20 mM cephalexin and benzylpenicillin. The net uptake rate for ampicillin was corrected for a diffusional component by subtracting a K_d value of 0.25±0.02 nmol · mg protein^{-1} · 30 s^{-1} · mM^{-1} from the total uptake rates. When the kinetics were transformed according to Hanes (Fig. 8), ampicillin uptake into BBMV as a function of substrate concentration revealed linearity, indicating presence of a single saturable uptake process. The apparent K_m value, as calculated from the Hanes plot, was 15.9±1.3 mM, and the apparent V_{max} was 6.01±0.27 nmol · mg protein^{-1} · 30 s^{-1}. The kinetics of ampicillin uptake in the presence of cephalexin or benzylpenicillin (Fig. 8) indicated competitive inhibition; the slopes (nmol · mg^{-1} · 30 s^{-1})$^{-1}$ were not significantly different from that of the control in the absence of inhibitors (0.166±0.10 for the control vs. 0.207±0.031 for benzylpenicillin and 0.235±0.011 for cephalexin).

Table III. Uptake of Ampicillin in Presence of Glycylglutamine and β-Lactam Antibiotics

	Uptake	Percent of control
	nmol · mg protein^{-1} · 30 s^{-1}	
Control	0.319±0.020	100
Gly-Gln	0.187±0.013*	58.6
Ampicillin	0.197±0.024*	61.7
Amoxicillin	0.171±0.018*	53.6
Benzylpenicillin	0.158±0.008*	49.5
Epicillin	0.229±0.019*	71.7
Cephalexin	0.194±0.027*	60.8
Cephamycin C	0.179±0.002*	56.1

Kidney BBMV were preloaded with a buffer with a pH of 7.4 (45 mM Hepes, 40 mM Mes, 40 mM Tris, and 300 mM mannitol). After preloading, BBMV were incubated for 5 s in the same buffer with pH of 7.4 containing 0.35 mM [^{3}H]ampicillin and 20 mM of either mannitol (control) or one of the lactam antibiotics listed above or glycylglutamine. Values represent the mean±SE of three preparations. * Significantly different from control ($P < 0.05$).

Discussion

Our study suggests that the transport of aminocephalosporins into renal BBMV is largely mediated by the oligopeptide/H^+ symporter. This suggestion is supported by several lines of evidence. First, both cephalexin and loracarbef competitively inhibited the uptake of glycylglutamine (Fig. 3). Second, like glycylglutamine, there was pH gradient–dependent uptake of cephalexin with a pronounced overshoot phenomenon (Fig. 5), which indicates that the transport of both glycylglutamine and cephalexin involves a proton symport. Third, the uptake of cephalexin as a function of concentration displayed saturation kinetics (Fig. 6) comparable to that of glycylglutamine studied under similar experimental conditions (11). Fourth, as shown in Fig. 7, there was a highly significant correlation in the potencies of various β-lactam antibiotics and peptides to inhibit uptake of [^{3}H]cephalexin and [^{3}H]glycylglutamine. The fact that patterns of inhibition of uptake of the radiolabeled substrates were identical further supports the conclusion that both share the same transporter.

Although there have been previous reports (8, 17) suggesting that oligopeptides and β-lactam antibiotics share a common transport system in kidney BBMV, this is the first study that establishes the structural features determining the affinity of these antibiotics for the binding site of the oligopeptide/H^+

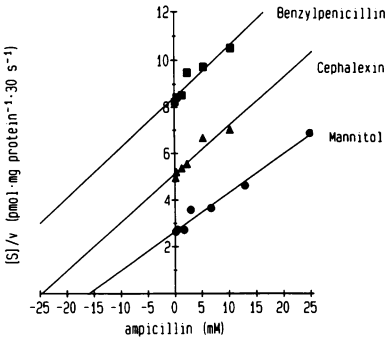

Figure 8. Hanes plots of ampicillin uptake, as a function of ampicillin concentration, in absence and presence of benzylpenicillin and cephalexin. BBMV were preloaded with a buffer with a pH of 7.4 (45 mM Hepes, 40 mM Mes, 40 mM Tris, and 300 mM mannitol). After preloading, BBMV were incubated for 30 s in the same buffer with a pH of 7.4 containing increasing concentrations (0.15–15 or 25 mM) of [^{3}H]-ampicillin and 20 mM concentration of either mannitol, cephalexin, or benzylpenicillin. Uptake rates represent mean±SE of three membrane preparations.

symporter. In particular, the study provides novel information on the importance of the α-amino group, configuration of NH_2 and COOH termini, and conformation for interaction of lactam antibiotics with the transporter.

Importance of the α-amino group in the D-phenylglycyl moiety. Our studies demonstrate that β-lactam antibiotics without an α-amino group do not interact with the symporter. The observation that the existence of an α-amino group is an essential feature for interaction with the substrate binding site of the carrier confirms our previous findings using di- and tripeptides as substrates (13). Oligopeptides with either blocked or substituted α-amino groups did not interact with the oligopeptide/H^+ symporter (13). The only exception found thus far is that metampicillin, in which the α-amino group is substituted by a related methylene amino group, retains some affinity (Table II).

It is possible that the failure of nonamino lactam antibiotics to interact with the oligopeptide/H^+ symporter could be related to the fact that they are anions, whereas the zwitterionic aminolactam antibiotics have no net charge at the pH used in our experiment. This possibility, however, does not seem likely. We have previously shown (13) that negatively charged dipeptides have a rather high affinity for the oligopeptide/H^+ symporter (apparent K_i values 150–300 μM).

Importance of the configuration of NH_2 and COOH termini. When we investigated the impact of NH_2- and COOH-terminal amino acid residues on the affinity of di- and tripeptides, it became apparent that hydrophobicity but not the molecular size of the amino acid residues determines the affinity. In fact, there was a significant correlation between the hydrophobicity of oligopeptides and their K_i value (13). The results of this study also indicate that an increase in the hydrophobicity of the NH_2 terminus (phenylglycyl chain) as well as of the COOH terminus (R_3 substituents) increases the affinity of the aminocephalosporins. The aminocephalosporins containing hydrophobic R_3-substituents like Cl and CH_3 had the highest affinity for the transporter. The hydrophilic and bulky R_3 substituent in cephaloglycin, for example, caused a > 20-fold decrease in affinity compared with the Cl residue in cefaclor. As judged by the comparison of cephalexin and cephradine and the various aminopenicillins, decreasing the polarity of the NH_2-terminal side chain also increases the affinity.

Conformation of β-lactam antibiotics. Although the oligopeptide/H^+ symporter preferentially binds oligopeptides containing L-α-amino acids, we previously found that this structural requirement can be modified (13). Dipeptides with hydrophobic NH_2-terminal D-amino acid residues displayed a high affinity (K_i values ranging between 50 and 150 μM) for the transporter (13). The present results are consistent with this observation. All the aminolactam antibiotics listed in Table II had a D-phenylglycylamido function, but still showed a rather high affinity for the oligopeptide/H^+ symporter.

Nevertheless, as demonstrated with D- and L-isomers of cephalexin and loracarbef, the interaction of the aminocephalosporins with the transporter also occurs stereospecifically. However, the difference in the K_i values of D- and L-cephalexin was lower than the difference between the K_i values of D- and L-loracarbef. This difference might be due to a difference in hydrolysis of these amino cephalosporins by kidney brush border membrane hydrolases. To investigate this possibility, we determined the extent of hydrolysis of D- and L-isomers of cephalexin and loracarbef (100 μM) after 1 min of incubation with kidney BBMV. We found that there was no hydrolysis of either D-cephalexin or D-loracarbef. On the other hand, there was a greater hydrolysis of L-cephalexin than of L-loracarbef (34±6 vs. 14±4%). Therefore, the apparent affinity of L-cephalexin may have been underestimated.

A similarity between the configurations of the β-lactam ring of cephalosporins and penicillins and the peptide bond of oligopeptides is that both have a planar structure. Although the thiazolidine and dihydrothiazine rings are rigid, there is some degree of rotational freedom around the C-N bond connecting the phenylglycyl chain and the lactam ring (18). This rotational freedom, which is equivalent to the rotational freedom along the α-carbons of oligopeptides, apparently allows enough flexibility for enabling lactam antibiotics to fit into and anchor at the binding site on the transporter. However, the fact that the conformation can be a limiting factor for a high-affinity interaction of β-lactam antibiotics with the oligopeptide/H^+ symporter is evident from the large difference between the affinities of loracarbef and epiloracarbef (Fig. 4). Computational analysis to determine bond angles revealed that the usual torsional angle of the C-N bond between the phenylglycyl chain and the lactam ring is in the range of $-180°$ to $-160°$ (18). This particular conformation seems quite important for the high affinity of aminolactam antibiotics, as evident from the low affinity of epiloracarbef. In epiloracarbef, the lactam ring structure faces in a usual manner below the plane of the paper.

Cephalosporins vs. oligopeptides. The apparent affinities of the aminocephalosporins studied in this experiment ranged between 45 and 1,560 μM (Table II). This is comparable to the range of affinities (10–500 μM) of 66 di- and tripeptides we previously studied. This similarity allows certain insights into the molecular structure and conformation that determine the affinity for the oligopeptide/H^+ symporter.

(*a*) The cephem nucleus of cephalosporins approximates the two COOH-terminal amino acid residues of tripeptides. In comparison to this structural aspect of tripeptides, the cephem nucleus represents a rather rigid structure and exists in only one conformation (18). However, the rigid structure of this ring does not appear to restrict the interaction of cephalosporins with the carrier site.

(*b*) The COOH-terminal peptide bond in cephalosporins is incorporated into the β-lactam ring and is, therefore, not free as in tripeptides. The fact that this structural feature is not important in overall affinity is consistent with the results of our

previous studies with a series of di- and tripeptides containing sarcosine (13). We found that modifications of the peptide bond nitrogen are crucial only when occurring in the first (NH_2-terminal) peptide bond. For example, methylation of the peptide bond nitrogen at this position decreased the affinity of a dipeptide four times, whereas the methylation of the COOH-terminal peptide bond nitrogen, as in Gly-Gly-Sar, did not alter the affinity compared with Gly-Gly-Gly. This indicated that alteration in the configuration of the COOH-terminal peptide bond of tripeptides is well accepted and does not decrease affinity. This observation might also apply to the present result, which shows that the incorporation of the peptide bond nitrogen into the β-lactam ring in cephalosporins does not reduce the affinity of these antibiotics compared with tripeptides.

Aminocephalosporins vs. aminopenicillins. One striking finding of our studies is that aminopenicillins have a much lower affinity than aminocephalosporins. Aminopenicillins and aminocephalosporins carry different substituents in their thiazolidine and dihydrothiazine rings, respectively. Penicillins are substituted generally with a dimethyl group at C_2 of the thiazolidine ring, whereas cephalosporins contain various R_3 substituents ranging from CH_3 to CH_2OCOCH_3. However, the difference in the configuration of ring substituents does not appear to account for the difference in affinity between penicillins and cephalosporins. The dimethyl group in penicillins is more hydrophobic than either CH_3 or CH_2OCOCH_3 in cephalosporins, and, therefore, aminopenicillins should theoretically have a higher affinity than observed, if hydrophobicity were the sole determinant of affinity.

It seems that the major structural difference between aminopenicillins and aminocephalosporins is the spatial position of the carboxyl group with respect to the β-lactam ring. The interatomic distances between the carboxyl group and the COOH-terminal peptide bond are significantly different in penicillins and cephalosporins (18). In the cephalosporins the carboxyl group is located almost planar to the cephem nucleus, whereas in the penicillins it faces below the planar thiazolidine ring. As a result, the interatomic distance between the carboxyl group and the carbonyl oxygen in penicillins is ~ 1 Å longer than in the cephalosporins (18). When measured in relation to the β-lactam ring carbonyl carbon, the distance in penicillins is shorter by ~ 0.5 Å. It is pertinent to note that the other interatomic distances, for example, the distance between the peptide bond nitrogen in the phenylacyl chain and the β-lactam ring carbonyl carbon, are not different in penicillins and cephalosporins.

The carboxyl oxygens are probably involved in electrostatic or dipole interactions with the substrate binding site of the transporter. We have previously shown that the free carboxy group in a substrate is important for a high-affinity interaction with the oligopeptide/H^+ symporter. Therefore, a novel finding of this study is that the spatial location of the carboxy group in relation to the other functional groups of the molecule is also quite important for substrate recognition and binding.

Evidence for a second transport system for β-lactam antibiotics. A major finding of this study was the discovery of another transport system, besides the oligopeptide/H^+ symporter, mediating the transmembrane flux of β-lactam antibiotics. The fact that this system is carrier-mediated is suggested by: (*a*) saturation in the uptake of ampicillin as a function of concentration and (*b*) competitive inhibition of uptake of ampicillin by other lactam antibiotics. Evidence that this system is distinctly different from the oligopeptide/H^+ symporter includes the following. (*a*) The transport activity of the electrogenic oligopeptide/H^+ symporter is dependent on the existence of an inwardly directed proton gradient, whereas ampicillin uptake was found to be unaffected by imposition of an inwardly directed H^+ gradient. (*b*) Various β-lactam antibiotics competed with the uptake of ampicillin whether or not they contained an α-amino group. In contrast, the oligopeptide/H^+ symporter is highly specific for substrates containing an α-amino group.

The fact that β-lactams without an α-amino group, like benzylpenicillin, inhibited only ampicillin uptake and not glycylglutamine uptake suggests that the recently identified transport protein in kidney BBMV (17) is related to the second transport system for β-lactam antibiotics described above. This suggestion is based on the following considerations.

The 127-kD protein was identified and characterized by photolabeling experiments with kidney and intestinal BBMV using benzylpenicillin and photoreactive azido analogues of Gly-Pro and cephalexin (17, 19–21). All three photoprobes labeled the same protein. Furthermore, substrate protection experiments with dipeptides, cephalexin, cefadroxil, and amoxicillin established the specificity of the incorporation of the label. However, it is pertinent to note that none of the photoprobes contained a free α-amino group; benzylpenicillin does not carry an α-amino group and the α-amino group of both cephalexin and Gly-Pro was blocked by attachment of the photoreactive azidobenzoate function. In view of our data on the substrate specificity of the transport system (13), it seems unlikely that the 127-kD protein labeled with these photoprobes represents the oligopeptide/H^+ symporter. As we have shown with oligopeptides as well as with the β-lactam antibiotics, a free α-amino group is a necessary feature for any interaction of a substrate with the carrier site. β-Lactam antibiotics without an α-amino group, regardless of their particular structure, as well as oligopeptides with a missing or blocked α-amino group (13), did not interact with the oligopeptide/H^+ symporter. The fact that the second transport system, identified by us using ampicillin as a probe, and the 127-kD protein both accept lactam antibiotics without an α-amino group suggests that they are related.

Conclusion. The results of this study suggest that the transport of zwitterionic α-aminocephalosporins is largely mediated by a system that has been characterized previously as the electrogenic oligopeptide/H^+ symporter in kidney BBMV. In comparison with α-aminocephalosporins, the α-aminopenicillins display much lower affinities. These differences in affinities seem to be determined by the substituents of the thiazolidine or dihydrothiazine ring and, in particular, by the sterical location of the carboxy group. On the other hand, penicillins, with or without an α-amino group, seem to share a low-affinity transport system with other β-lactam antibiotics and oligopeptides. This system operates in the absence of a transmembrane pH gradient and is less discriminative with respect to the structural features of its substrates. These results may have application in the design of new lactam antibiotics, which are continually being introduced as therapeutic agents.

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