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BINDING OF AMINO β-LACTAM ANTIBIOTICS TO SOLUBLE PROTEIN FROM RAT INTESTINAL MUCOSA—I

PURIFICATION OF DRUG-BINDING PROTEIN

KEN ISEKI, KEN-ICHI MORI, KATSUMI MIYAZAKI and TAKAICHI ARITA
Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan

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Abstract—Several amino β-lactam antibiotics, including ampicillin, amoxicillin, cyclacillin, cephalexin, cephradine and cefadroxil, were found to bind *in vitro* to specific components in 105,000 *g* supernatant of homogenate obtained from rat intestinal mucosa. The major binding component (fraction b) was purified by chromatography on DEAE-cellulofine and by gel filtration on Sephadex G-50. The molecular weight of fraction b was determined by SDS polyacrylamide gel electrophoresis (15,000 Da).

The binding behaviour of these amino β-lactam antibiotics to fraction b were estimated by equilibrium dialysis. There were significant high affinities of all tested amino β-lactam antibiotics which were well absorbed from intestine, but there was not a good correlation between binding and absorption of these drugs. It was also found that poorly absorbed cephalosporins which lack aminobenzyl group in their structure, cefazolin and cephaloridine, did not bind to fraction b.

Amino β-lactam antibiotics, widely used antimicrobial agents, are well absorbed from the intestinal lumen after oral administration despite their poor lipophilicity. The mechanisms of intestinal absorption of these drugs have been extensively investigated [1–8]. Some reports [1–5] suggested that a carrier-mediated transport system for dipeptides or amino acids, which have zwitterionic structure like these drugs, participates in the membrane transport of several amino β-lactam antibiotics. However, Yamashita *et al.* [9, 10] suggested that the capacity and affinity of ampicillin, cephalexin and cephradine to such transport systems are extremely low compared to those of L-phenylalanine, and that intracellular accumulation processes are involved in their transport system using kinetic analysis [10]. Our previous report [5] showed that intestinal tissue concentrations of cephalexin and cephradine became higher than those in the mucosal solution by using everted rat intestinal sac preparations. We also demonstrated that there was a protein fraction named F1, which possesses the specific binding properties for several amino β-lactam antibiotics in the 105,000 *g* supernatant of homogenates obtained from rat small intestinal mucosa [11]. From these implications, it would be possible to consider that this intracellular accumulation process plays some roles in the absorption systems of these amino β-lactam antibiotics. Moreover, despite many investigations, the common properties of intestinal absorption to these drugs, aminopenicillins and aminocephalosporins, remain unknown.

The present work was undertaken to clarify the affinity characteristics of several amino β-lactam antibiotics, including ampicillin, amoxicillin, cyclacillin, cephalexin, cephradine and cefadroxil, to the intestinal mucosa. A major binding component for these drugs has been purified, and the binding behaviour of these drugs and monobasic cephalosporins which lack aminobenzyl group in the structure, cefazolin, cephaloridine, were examined.

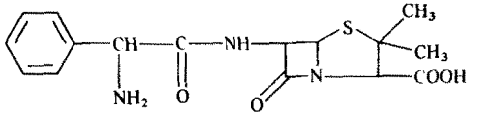
MATERIALS AND METHODS

Materials

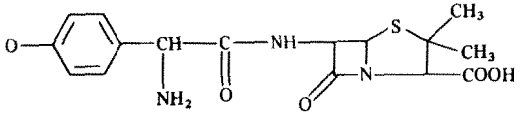
Ampicillin anhydrous, cyclacillin anhydrous (Takeda Chemical Industries, Osaka, Japan, respectively), amoxicillin trihydrate (Kyowa Hakko Kogyo Co., Tokyo, Japan), cephalexin monohydrate, sodium cephaloridine (Shionogi & Co., Tokyo, Japan), cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan) and cefadoroxil (Banyu. Co., Tokyo, Japan) were kindly donated. The structures of β-lactam antibiotics tested are illustrated in Fig. 1.

SDS-PAGE Marker III as the standard protein for the determination of molecular weight were obtained from Fluka AG, Chemische, Fabrik, Switzerland. Sephadex G-75 (super fine) and Sephadex G-50 (fine) were purchased from the Pharmacia Fine Chemical Co. Ltd. (Uppsala, Sweden). DEAE-cellulofine (AM type) were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). All the chemicals were used without further purification. Cells for the equilibrium dialysis method were obtained from Abe Kagaku Co. Ltd. (Tokyo, Japan). Dialysis tubing (Spectrapor 6, MWCO 2000 and MWCO 8000) were purchased from Spectrum Medical Industries Inc. (Los Angeles, CA). All other reagents used for the experiments were of the highest purity available.

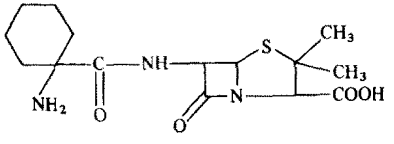
Zwitterionic β-lactam antibiotics



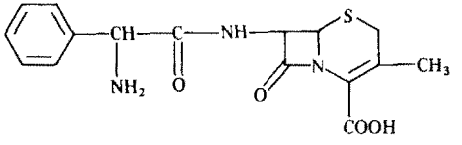
Ampicillin



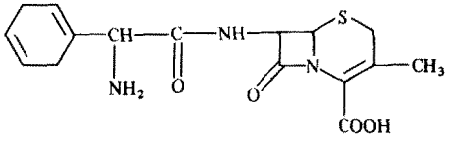
Amoxicillin



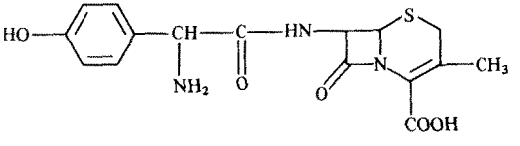
Cyclacillin



Cephalexin

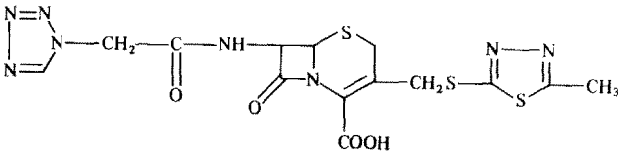


Cephradine

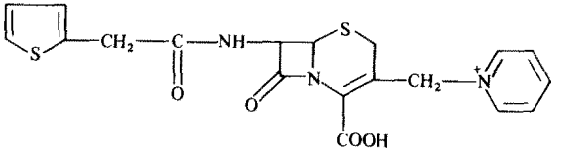


Cefadroxil

Monobasic β-lactam antibiotics



Cefazolin



Cephaloridine

Table 1. Purification table of cephalexin-binding protein (fraction b) in the rat intestinal soluble fraction

Purification step	Total protein (mg)	Yield (%)	Specific activity (nmol/mg)	Purity
105,000 *g* sup.	305.2	100	1.56	1
DEAE-cellulofine (G2 fraction)	5.61	19.4	16.47	10.6
Lyophilization	4.76	18.5	18.49	11.9
Sephadex G-50 (fraction b)	0.52	18.0	168.0	107.7

Specific intensity were measured by equilibrium dialysis using the dialysis cell. The concentration of protein was determined by the method of Lowry *et al.* [12].

Animals

Male Wistar rat (230–300 g) (Inst. Animal Exp., School of Med., Hokkaido Univ., Sapporo, Japan) were used as the intestine source.

Preparation of drug binding protein

All procedures were performed in the cold room (4°) or on ice.

(1) *Preparation of the supernatant fraction.* A 105,000 *g* supernatant fraction was prepared from a 20% homogenate of the intestinal mucosa from eight rats as described previously [11]. About 50 cm length of the small intestine below the pylorus was removed, rinsed with cold saline, everted, and rinsed again with cold saline and blotted with a filter paper for the complete removal of mucus. The mucosa was scraped off gently with a glass slide, and was homogenized in 10 mM phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 8.0 (buffer A), using a motor-driven, Teflon pestle, glass homogenizer. The homogenate was centrifuged at 105,000 *g* for 1.5 hr at 4°.

(2) *DEAE-cellulofine ion-exchange chromatography.* The 50 ml of supernatant fraction from intestinal mucosa homogenates, exclusive of floating fat, was applied to a column of DEAE-cellulofine (i.d. 1.6 × 70 cm) equilibrated with buffer A. After eluting the column with 350 ml of buffer A, a step wise gradient composed of 400 ml of buffer A containing 0.5 M KCl was used to elute the column. Aliquots of 4.0 ml were collected at a flow rate of 40 ml/hr. The elution pattern was monitored spectrophotometrically at 280 nm and protein concentration was determined by Lowry's method [12].

(3) *Sephadex G-50 gel filtration.* The fraction (120 ml) from (2) was concentrated to 30 ml by lyophilization and dialyzed against 10 mM phosphate buffer containing 0.1 M NaCl, pH 7.4 (buffer B), using the dialysis tubing (Spectrapor 6, MWCO 2000). The concentrated sample was centrifuged at 20,000 *g* for 30 min; the clear supernatant was chromatographed on a Sephadex G-50 column (i.d. 2.5 × 65 cm) which was equilibrated with buffer B. Elution was performed with buffer B at the flow rate of 10–12 ml/hr, and 4.0 ml of fractions were collected. The elution pattern was monitored spectrophotometrically at 215 nm and 280 nm, and protein concentration was determined by Lowry's method. The fractions were dialyzed against three changes (each of 100 vol.) of distilled water using the dialysis tubing (Spectrapor 6. MWCO 2,000) and lyophilized.

Binding experiments by equilibrium dialysis

The protein samples were previously dialyzed against buffer B. The lyophilized samples were dissolved in the same buffer. The binding studies were performed at 37° in cells each of which consisted of two half cells (volume 2.08 ml; membrane surface area 4.15 cm^2) separated by a membrane (Spectrapor 6. MWCO 8000).

In preliminary experiments, the time required for cephalexin, cephradine, cefadroxil, cefazolin, cephaloridine, cyclacillin, amoxicillin and ampicillin dialysed against buffer B to reach equilibrium were determined, and in all cases equilibrium was reached by 3 hr. At the end of equilibrium-dialysis experiments (7 hr), samples were taken from both sides of the dialysis membrane, and used for the determination of the concentration of drugs.

Electrophoresis

Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis was carried out by the method based on that of Swank and Munkres [13]. The concentration of acrylamide was 12.5% and the ratio of acrylamide and *N,N'*-methylene bis acrylamide was 20:1 in the presence of 8.0 M urea. Prior to electrophoresis, the proteins were heated to 70° with 1% mercaptoethanol for 10 min. After electrophoresis, gels were removed, the tracking dye (brilliant green) band suitably marked and the gels stained for 2–3 hr in 0.25% Coomassie Brilliant Blue R-250 dissolved in a solution containing water (5 parts), acetic acid (1 part), and methanol (5 parts).

Analytical method

For the determination of β-lactam antibiotics, high performance liquid chromatography was used. A high performance liquid chromatograph, Hitachi 638-50, equipped with variable-wavelength u.v. monitor (638-41) was used. The reversed phase column was packed with Hitachi Gel #3053 (ODS, 5 μm, i.d. 4 × 250 mm, Hitachi Ltd., Tokyo, Japan) for the determination of cephalexin, cephradine, ampicillin, cefazolin and cyclacillin or Hitachi Gel #3056 (ODS, 5 μm, i.d. 4 × 250 mm, Hitachi Ltd., Tokyo, Japan) for cephaloridine. For the analysis of amoxicillin and cefadroxil, Zorbax-ODS (5 μm, i.d. 4.6 × 250 mm, Shimazu Ltd., Kyoto, Japan) was used. The mobile phase was the mixture of methanol–0.1 M acetate for the determination of cephalexin, cephradine and cefazolin (15:85 by volume, pH 6.0), cephaloridine (30:70 by vol.,

pH 6.8). In the case of the other drugs, the mixture of methanol–0.05 M K_2HPO_4 solutions adjusted to pH 6.0 with 4 M sodium hydroxide solution was used for the determination of ampicillin and cefadroxil (10:90 by vol.). The flow rate was maintained at 0.7 ml/min, and the column was warmed at 55° using a water bath circulator. The wavelength of the monitor was set at 210 nm and 260 nm for penicillins and cephalosporins, respectively.

In regular assay of the drugs in the sample solution of equilibrium dialysis, 2 ml of methanol was added to 1 ml of the sample solution, and then centrifuged at 3000 *g* for 20 min. An appropriate volume of the supernatant was injected into the liquid chromatograph. The drug concentrations were calculated from the peak height ratios using calibration curve, and the internal standard was selected from the drugs tested in these experiments.

RESULTS

Preparation of drug binding protein

A summary of the purification step is presented in Table 1.

DEAE-cellulofine ion exchange chromatography of rat intestinal 105,000 *g* supernatant yielded in elution pattern as shown in Fig. 2. Six peaks of elution (G1–G6) monitored spectrophotometrically at 280 nm were obtained. A higher binding activity for cephalexin was obtained in the second protein fraction (G2 fraction).

Results of further chromatography of G2 fraction on Sephadex G-50 gel filtration are shown in Fig. 3. Three fractions (fraction a, b and c) were pooled and the binding activity for cephalexin, shown by the vertical bars, were determined from equilibrium dialysis. The fraction b had the highest binding activity, but other fractions had little activity, as shown in Fig. 3. Furthermore, one peak was obtained from the rechromatography on Sephadex G-50 gel filtration of fraction b.

On the final step of purification, specific activity of cephalexin-binding was enhanced 100-fold compared to supernatant fraction from rat intestinal mucosa, and yield was 18.0%.

Electrophoresis of the fraction b and molecular weight determination

The purity and molecular weight of purified fraction b were estimated by SDS–polyacrylamide gel electrophoresis. The fraction b migrated as a single band, and the molecular weight of this fraction were 15,000 Da.

Binding behaviour of several β-lactam antibiotics to fraction b

Figure 4 shows the binding behaviour of several β-lactam antibiotics to fraction b using equilibrium dialysis at 20 μM of drug concentration. In these binding experiments, the binding of the amino β-lactam antibiotics was significantly higher, while monobasic β-lactam antibiotics such as cefazolin and cephaloridine which are poorly absorbed from the intestine had no binding to fraction b.

DISCUSSION

The experiments described show the presence of a protein factor in rat intestinal 105,000 *g* supernatant able to bind amino β-lactam antibiotics. Although exhaustive criteria of protein purity were not applied, SDS–polyacrylamide gel electrophoresis and rechromatography of fraction b on Sephadex G-50 demonstrated the presence of only a single protein band in each experiment.

In earlier papers [5, 11], we have shown that amino β-lactam antibiotics were accumulated in the tissue wall and the uptake and accumulation were proportional to the degree of absorption in rat intestine. We have also shown that there was a highly significant binding fraction (F1) of these antibiotics from intestinal cytosol fraction using Sephadex G-75 gel filtration [11]. In preliminary work, F1 fraction on Sephadex G-75 chromatography and fraction b obtained from the present study were eluted to the same position on Sephadex G-50 chromatography, and a high cephalexin binding activity was obtained from both fractions (data not shown). This finding indicates that fraction b was a major component of the F1 fraction found in our previous study.

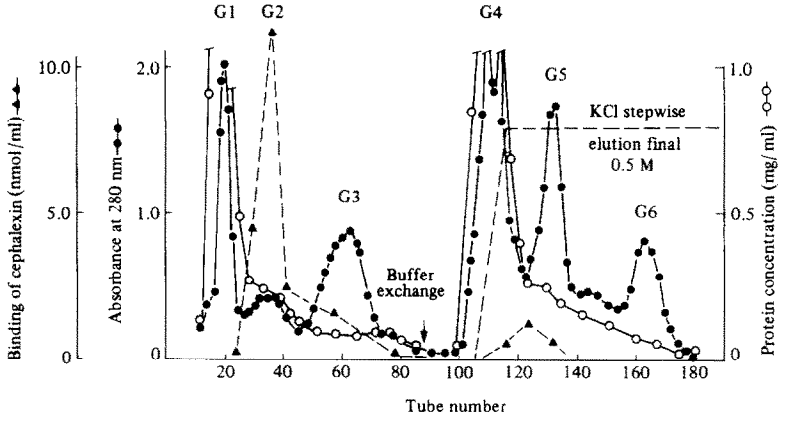


Fig. 2. DEAE cellulofine ion exchange chromatography of rat intestinal 105,000 *g* supernatant. Equilibration buffer was 10 mM phosphate buffer (pH 8.0). Flow rate was 40 ml/hr and 4.0 ml of fractions were collected. Protein was measured by Lowry's method and cephalexin binding activity was determined by equilibrium dialysis method.

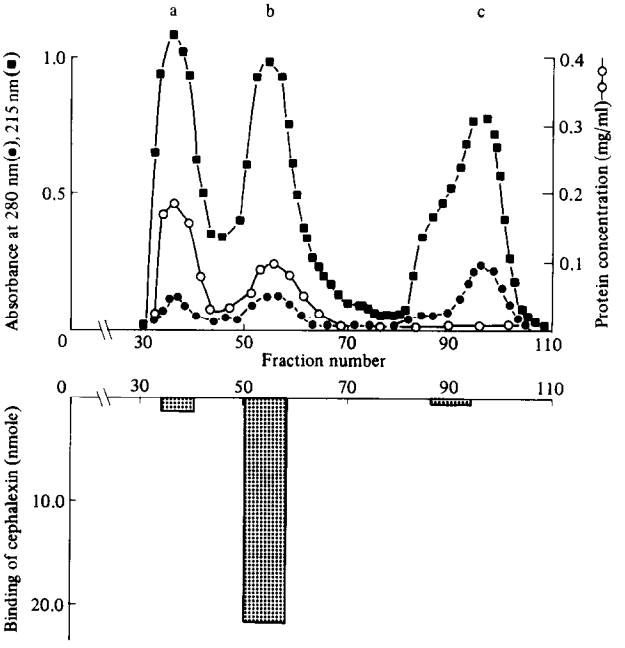


Fig. 3. Gel filtration chromatography of G2 fraction from DEAE ion exchange chromatography of rat intestinal 105,000 *g* supernatant on Sephadex G-50. Flow rate was 10–12 ml/hr and the effluent was 10 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl. The effluent was collected in 4.0 ml fractions. Protein was determined by Lowry's method.

From the binding experiments of several β-lactam antibiotics to fb fraction, there was a specific binding of amino β-lactam antibiotics which have the zwitterionic structure and were well absorbed from intestine. There are many publications which concern the absorption of β-lactam antibiotics from small intestine, and much interests have been focused on the intestinal absorption mechanisms of amino β-lactam antibiotics. Saturation phenomena and the carrier-mediated transport system for cephalexin, cephradine, cefadroxil and cyclacillin have been reported from a view of competitive inhibition of these drugs [14–16]. Our previous report [17, 18] also demonstrated that there was the mutual inhibition between these amino β-lactam antibiotics in the absorption from intestinal lumen and the accumulation to intestinal tissue. Yamashita *et al.* [10] have investigated the transport characteristics of β-lactam antibiotics in rat jejunum using the electrophysiological technique *in vitro*, and they found that each saturable component was present in the transport process of rat intestinal cell (influx rate into the epithelial cell from the mucosal side, and accumulation to tissue). They also reported that the K_m (saturation constant) value of influx rate were apparently smaller than those of accumulation in the case of both cephalexin and cephradine. From these implications and the results obtained in these experiments, it is suggested that this high binding to fraction b explains the accumulation to the intestinal mucosa of these amino β-lactam antibiotics, and it is possible that this process plays some role in the transfer of these drugs.

Previously, we reported that the absorption of amino β-lactam antibiotics were in the order of cephalexin ≧ cephradine > amoxicillin > ampicillin [11]. In the present study, however, the degree of binding to fraction b was not necessarily in agreement with absorption of all amino β-lactam antibiotics (Fig. 4). Because of this discrepancy it is conceivable that the condition of binding experiments was not different from the intrinsic state of fraction b in intestinal epithelial cells.

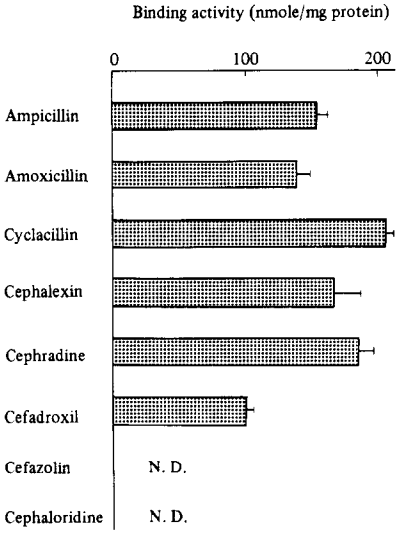


Fig. 4. Binding behaviour of β-lactam antibiotics to fraction b. Results are expressed as mean of 6–12 experiments with SEM. N.D., not detected.

More detailed characteristics of the binding of these antibiotics to fraction b, which was associated with absorption mechanisms, are under investigation from the point of view of the maximum amount of binding and dissociation constant or mutual inhibition among these antibiotics.

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