Biochemical Pharmacology, Vol. 36, No. 11, pp. 1843–1846, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
© 1987. Pergamon Journals Ltd.

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

MUTUAL INHIBITION OF BINDING AMONG AMINO β-LACTAM ANTIBIOTICS AND BINDING CHARACTERISTICS

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

(*Received* 23 *September* 1986; *accepted* 11 *December* 1986)

Abstract—The characteristics of binding of amino β-lactam antibiotics including ampicillin, amoxicillin, cephalexin and cephradine to fraction b obtained from the 105,000 *g* supernatant of rat small intestinal mucosa was investigated.

The mutual inhibition of binding among these antibiotics was observed, and these were dependent on the concentration of inhibitors. It was found that dipeptides such as L-carnosine and glycylglycine significantly reduced the binding of cephalexin to fraction b, but the binding of cephradine was only slightly decreased by these dipeptides. Furthermore, the binding of cephalexin and cephradine was not influenced by amino acids (L-phenylalanine, glycine).

Although ANS(1-anilino-8-naphthalenesulfonic acid magnesium salt), which is a hydrophobic probe, bound to the fraction b, there was no competitive inhibition in binding between ANS and amino β-lactam antibiotics.

The Scatchard plot of binding data of cephalexin gave two dissociation constant (K_d) values (1.37 and 15.7 μM). On the other hand, one K_d value (11.2 μM) was obtained for ampicillin.

In our previous report [1], soluble protein as binder of several β-lactam antibiotics was purified from the 105,000 *g* supernatant of rat small intestine. We found that amino β-lactam antibiotics, which have zwitterionic structures and are well absorbed from intestine, have a high binding affinity to this protein fraction (fraction b). This protein was purified from the soluble fraction of small intestinal mucosa homogenate using DEAE ion exchange chromatography and Sephadex G-50 gel filtration, and molecular weight was approximately 15,000 Da.

In the present study, more detailed binding characteristics of these antibiotics to fraction b have been investigated.

MATERIALS AND METHODS

Materials

Ampicillin anhydrous (986 μg/mg), cyclacillin anhydrous (1000 μg/mg) (Takeda Chemical Industries, Osaka, Japan, respectively), cephalexin monohydrate (947 μg/mg), cephaloridine (976 μg/mg) (Shionogi & Co., Osaka, Japan), cefazolin (969 μg/mg) (Fujisawa Pharmaceutical Co., Osaka, Japan), amoxicillin trihydrate (856 μg/mg) (Kyowa Hakko Kogyo Co., Tokyo, Japan), cephradine dihydrate (960 μg/mg) (Sankyo Co., Tokyo, Japan) were kindly donated. ANS (1-anilino-8-naphthalenesulfonic acid magnesium salt) was purchased from Nakarai Chemicals Ltd., Kyoto, Japan. L-Phenylalanine, glycine and glycylglycine were purchased from Sigma Chemical Co., St. Louis, MO. L-Carnosine was from Fluka AG, Chemische Fabrik, Switzerland. All the reagents were of special grade and used without further purification.

Fraction b was isolated and purified as described previously [1].

Binding studies with fraction b

Equilibrium dialysis. Fraction b (lyophilized samples) were dissolved in 10 mM phosphate buffer containing 0.1 M NaCl, pH 7.4 (buffer B in ref. 1). The binding studies of drugs to fraction b were performed at 37° by the method of equilibrium dialysis using the dialysis cells described previously [1]. The inhibition experiments using drugs, amino acids and dipeptides were performed by addition of inhibitory compounds to the both sides of the dialysis cells.

Determination of binding for ANS by fluorescence intensity. Binding studies for ANS were performed according to the method of Sugiyama *et al.* [2]. To the cuvettes containing 2 ml of fraction b in buffer B, varying volumes (up to 40 μl) of ANS stock solution (5 mM) were added. The solution was stirred with a micro-magnetic stirrer after each addition and the fluorescence intensity was measured at 480 nm (excited at 400 nm) using a Hitachi 650-60 fluorescence spectrophotometer. A cell-holder was warmed at 25° or 37° using a water bath circulator. The apparent dissociation constant (K_d) was calculated from the modified Scatchard plot namely the plot of the change in fluorescence (ΔF)/[ANS] vs ΔF. Influences of several amino β-lactam antibiotics to

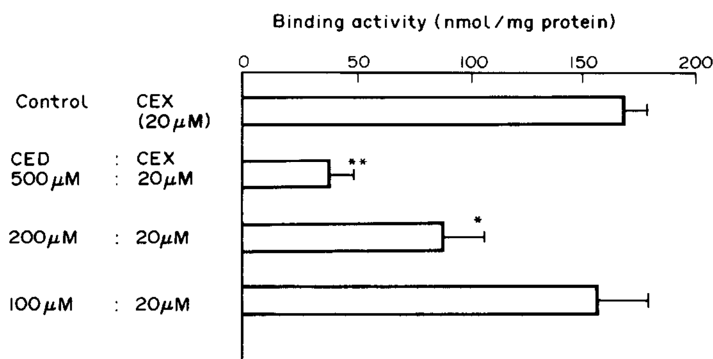

Fig. 1. Effects of various concentrations of cephradine on the binding of cephalexin to fraction b. Results are expressed as mean of 3–4 experiments with SEM. Binding activity was measured by equilibrium dialysis. CEX: cephalexin, and CED: cephradine. *P < 0.05; **P < 0.01.

ANS binding were determined using competitive techniques by following the decrease of the fluorescence. To the cuvettes containing 2 ml of mixture of 79.3 μg/ml fraction b, and 12.5 μM ANS in the buffer B, varying volumes (up to 40 μl) of the drug solution (26.6 mM) were added. The solution was mixed after each addition, and the fluorescence was measured as described above.

Analytical method

β-Lactam antibiotics were analyzed by a high performance liquid chromatograph, Hitachi 655 (Hitachi Ltd., Tokyo, Japan) equipped with variable-wavelength u.v. detector (638-41) as described previously [1]. Protein was measured by the method of Lowry [4] with bovine serum albumin as standard.

RESULTS

Mutual binding inhibition between amino β-lactam antibiotics

The inhibition behaviour among various amino β-lactam antibiotics to fraction b was examined to clarify whether the binding site of fraction b is common for these drugs. In order to estimate the inhibitory effect of cephradine on the cephalexin binding, first, the binding amounts of cephalexin were measured in the presence of various concentrations of cephradine. The result was shown in Fig. 1. The significant inhibitory effect of cephradine on the binding of cephalexin (20 μM) was observed at more than 200 μM of cephradine, and the degree of inhibition was proportional to the concentration of

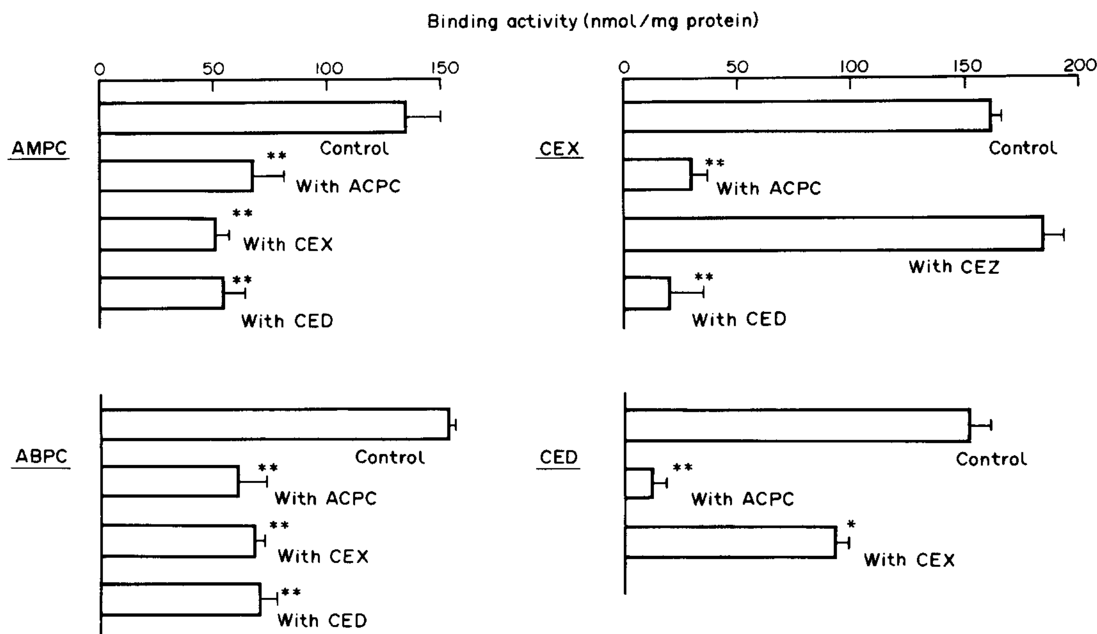

Fig. 2. Inhibition behaviour among amino β-lactam antibiotics. Results are expressed as mean of 3–8 experiments with SEM. Binding activity was measured by equilibrium dialysis. ABPC: ampicillin, AMPC: amoxicillin, ACPC: cyclacillin, CEX: cephalexin, CED: cephradine, and CEZ: cefazolin. The concentration of drugs or inhibitors was 20 or 500 μM, respectively. *P < 0.05; **P < 0.01.

cephradine which was added to both sides of dialysis cells.

Subsequently, to investigate whether the competitive inhibition observed between cephalexin and cephradine was present among another β-lactam antibiotics, the inhibition behaviours among these drugs were studied. As the results in Fig. 2 show, the binding of ampicillin, amoxicillin, cephradine and cephalexin (20 μM) to fraction b were reduced significantly by the presence of another amino β-lactam antibiotics at 500 μM concentrations which are selected based on the result shown in Fig. 1.

In contrast, however, there was no influence of cefazolin, which was poorly absorbed from intestine and showed no binding behaviour [1], on the binding of cephalexin to fraction b (Fig. 2). These results suggested that there was a common binding site among these amino β-lactam antibiotics to fraction b, but not monobasic cephalosporins.

Binding property of amino β-lactam antibiotics to fraction b

In order to clarify the binding specificity of these drugs to fraction b, the influences of amino acids and dipeptides as zwitterionic compound, or ANS as hydrophobic probe on drug-binding to this fraction were studied. L-Phenylalanine, glycine, L-carnosine and glycylglycine were used to examine whether amino and carboxyl groups were required for binding specificity of substrate. The results for cephalexin and cephradine are shown in Table 1. There was significant inhibition in binding between cephalexin and dipeptides (500 μM), but the binding of cephradine was slightly reduced by the presence of these dipeptides. On the other hand, there were no comparable change in the binding of both cephalosporins in the absence or presence of L-phenylalanine or glycine.

To clarify specificity of the affinity to fraction b in detail, ANS, which has been studied as a hydrophobic probe on proteins [5–7] or membranes [8, 9], were used. ANS bound to fraction b and dissociation constant (K_d) were calculated to 23 μM from the slope of Scatchard plot (Fig. 3).

However, there were no changes in the binding of ANS to fraction b in the presence of several β-lactam antibiotics including amino and monobasic cephalosporins over the drug concentration range 0–500 μM. Moreover, the binding of amino β-lactam antibiotics to fraction b was not affected by the presence of ANS (data not shown). These results suggest, therefore, that there were different binding sites to fraction b between the amino β-lactam antibiotics and ANS.

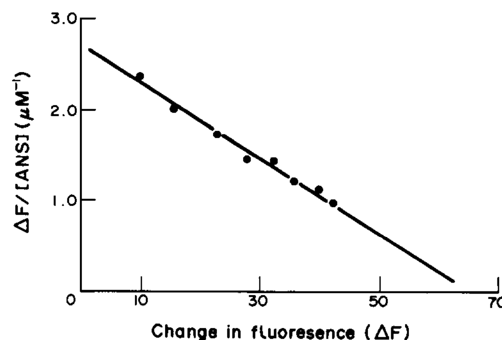

Fig. 3. Scatchard plot of ANS binding to fraction b. Binding of ANS was determined by fluorescence intensity change (ΔF). Each point represents the mean of two experiments. ANS concentration varied from 5 to 30 μM, while the concentration of fraction b (79.3 μg/ml) were held constant. The experiments were performed at 25°.

Stoichiometry of the binding characteristics of drugs

The stoichiometry of the binding of cephalexin or ampicillin to fraction b was determined by the equilibrium dialysis method. The concentration of fraction b was 8.8 μM and the concentrations of drugs in the buffer solution were from 3 to 100 μM. Scatchard plot of the binding as a function of cephalexin concentration gave two apparent K_d values (1.37, 15.7 μM), low and high affinity (Fig. 4a). On the other hand, the K_d value of ampicillin (11.2 μM) was the only one which corresponded to the low affinity of cephalexin binding (Fig. 4b).

DISCUSSION

In this study, we observed that there were significant mutual inhibition among amino β-lactam antibiotics, and it is considered that there are the common binding site(s) for amino β-lactam antibiotics to fraction b. In the previous papers, we showed that the degree of intestinal absorption of amino β-lactam antibiotics (ampicillin, amoxicillin, cephalexin and

Table 1. Effects of dipeptides (L-carnosine, glycylglycine) and amino acids (glycine, L-phenylalanine) on cephalexin and cephradine binding to fraction b

Inhibitor	Concentration (μH)	Binding activity (nmol/mg protein)	
		Cephalexin	Cephradine
None	—	165.8 ± 8.0	163.4 ± 17.4
L-Carnosine	500	72.0 ± 12.1**	133.8 ± 30.1
Glycylglycine	500	65.5 ± 11.5**	138.4 ± 29.6
L-Phenylalanine	500	175.2 ± 17.8	173.0 ± 8.4
Glycine	500	158.7 ± 23.3	—

Results are expressed as mean of 3–6 experiments with SEM.

The equilibrium dialysis method was used for the determination of binding activity. The concentrations of cephradine and cephalexin were 20 μM.

** $P < 0.01$.

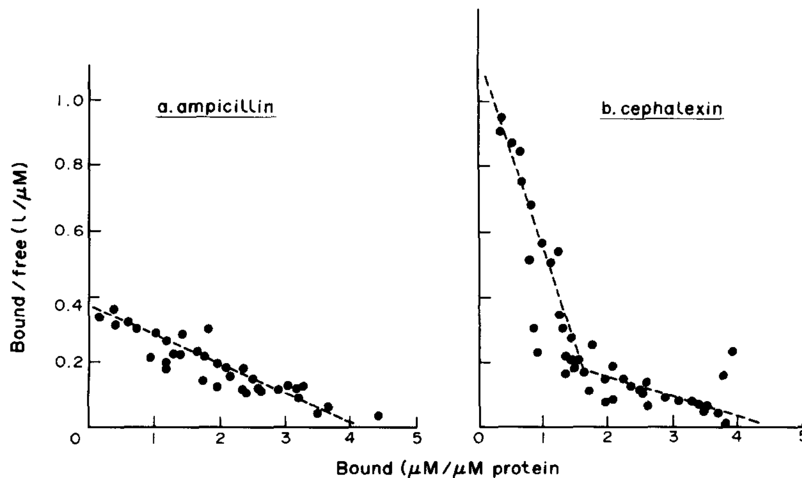

Fig. 4. Scatchard plot of ampicillin (panel a) and Cephalexin (panel b) binding to fraction b. The concentration of ampicillin or cephalexin was 3–100 μM. Binding of drugs were determined by equilibrium dialysis.

cephradine) was proportional to the amount of drug in the intestinal tissue [10], and that the mutual inhibition in their intestinal absorption was observed among these antibiotics [3, 11]. Furthermore, we also indicated that all amino β-lactam antibiotics tested, which are well absorbed from intestine, had high affinities to fraction b compared to the monobasic cephalosporins such as cefazolin and cephaloridine [1]. From these previous [1, 3, 10, 11] and present results, it seems that these antibiotics have similar binding characteristics, and this common binding property plays an important role in the absorption process of these drugs from the intestine. Nakashima *et al.* [12, 13] have reported that the dipeptides inhibited the absorption of amino cephalosporins, and indicated that the transport system of these drugs was associated with the carrier-mediated transport system of dipeptide. On the contrary, it has been reported in another paper [14] that the absorption of aminopenicillins (ampicillin and amoxicillin) was not affected by the presence of dipeptides. Therefore, it is difficult to think that the dipeptide transport system is the common major mechanism for the absorption of these amino β-lactam antibiotics.

The Scatchard plot of the cephalexin binding to fraction b indicated two types of binding sites, but ampicillin had only a lower affinity site. The result may explain the reason for the discrepancies obtained previously between the degree of binding to fraction b and the absorption of these antibiotics [1], in which the binding had been examined using lower drug concentration at 20 μM. Namely, in the native condition, it appears that cephalexin, which is absorbed more than ampicillin, occupies high and low affinity sites of fraction b during the absorption process, but, ampicillin has only a low affinity site.

In this report, we also examined the binding characteristics of fraction b by studying not only mutual inhibition among amino β-lactam antibiotics but also the effect of monobasic cephalosporins, dipeptides, amino acids and ANS on the binding of these amino β-lactam antibiotics. Although the reason for the difference observed in the effect of dipeptides on cephalexin and cephradine (Table 1) should be further examined, it is suggested that both amino and carboxyl groups which are adequately distant from each other in the molecular structure are associated with the affinity to fraction b. Further studies for the role of fraction b related to cellular transport of these amino β-lactam antibiotics and biological function are under examination.

Acknowledgements—The authors wish to thank Mrs Mie Miyamoto and Mr Takuya Furukawa for their technical assistance in this work.

REFERENCES

1. K. Iseki, K. Mori, K. Miyazaki and T. Arita, *Biochem. Pharmac.* **36**, 1765 (1987).
2. Y. Sugiyama, T. Iga, S. Awazu and M. Hanano, *Biochem. Pharmac.* **29**, 2063 (1980).
3. K. Iseki, A. Iemura, H. Sato, K. Sunada, K. Miyazaki and T. Arita, *J. Pharmacobio-Dyn.* **7**, 768 (1984).
4. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
5. C. F. Chingnell, *Ann. N.Y. Acad. Sci.* **226**, 44 (1973).
6. Y. Sugiyama, A. Stolz, J. M. Hershman and N. Kaplowitz, *Biochim. biophys. Acta* **801**, 184 (1984).
7. K. Zierler and E . Rogus, *Biochim. biophys. Acta* **514**, 37 (1978).
8. M. B. Feinstein, L. Spero and H. Felsenfield, *FEBS Lett.* **6**, 245 (1970).
9. D. H. Haynes and H. Staerk, *J. Membr. Biol.* **17**, 313 (1974).
10. K. Umeniwa, O. Ogino, K. Miyazaki and T. Arita, *Chem. Pharm. Bull.* **27**, 2177 (1979).
11. K. Miyazaki, K. Ohtani, K. Umeniwa and T. Arita, *J. Pharmacobio-Dyn.* **5**, 555 (1982).
12. E . Nakashima, A. Tsuji, H. Mizuo and T. Yamana, *Biochem. Pharmac.* **33**, 3345 (1984).
13. E . Nakashima and A. Tsuji, *J. Pharmacobio-Dyn.* **8**, 623 (1985).
14. T. Kimura, H. E ndo, M. Yoshikawa, S. Muranishi and H. Sezaki, *J. Pharmacobio-Dyn.* **1**, 768 (1979).