ENZYMATIC STUDIES ON THE MECHANISM OF ACTION OF CEFOXITIN

CORRELATION BETWEEN THE AFFINITIES OF CEFOXITIN TO PENICILLIN-BINDING PROTEINS AND ITS RATES OF INHIBITION OF THE RESPECTIVE PENICILLIN-SENSITIVE REACTIONS IN *E. COLI*

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The affinities of cefoxitin, a cephamycin antibiotic, to penicillin-binding proteins of *Escherichia coli* were reexamined using a recently developed method for separating penicillin-binding proteins. The inhibitions by this antibiotic of four measurable penicillin-sensitive enzymatic reactions, the reactions of D-alanine carboxypeptidases IA and IB, cross-bridge formation and concomitant release of D-alanine, were also measured. An approximate correlation was found between the affinities of cefoxitin to the penicillin-binding proteins responsible for these reactions and its rates of inhibition of the respective penicillin-sensitive reactions.

Cefoxitin (CFX) is a cephamycin antibiotic with a methoxy group at the 7α-position of the cephalosporin skeleton[1] and, like many other β-lactam antibiotics, it inhibits cell wall peptidoglycan synthesis, causing lysis of the cells. It is very resistant to β-lactamases. The binding affinities of cefoxitin to membrane proteins of *Escherichia coli* (penicillin-binding proteins, PBPs) have been investigated by SPRATT[2] and its binding to PBPs of *Proteus* species[3], and *Pseudomonas aeruginosa*[4] have also been studied. Cefoxitin shows very high affinities to almost all the PBPs of the bacteria mentioned above, but it has low affinity to PBP-2, and also to PBP-4′ of *E. coli*, to which mecillinam, an amidinopenicillin, binds exclusively. Information has recently been obtained on the functions of PBPs or their correspondence to known D-peptide-transferring enzymes, such as D-alanine carboxypeptidases, by isolating mutants lacking one of the PBPs. This paper describes the correlation between the high affinities of cefoxitin to each PBP in *E. coli* and its high potency to inhibit the respective enzyme reactions. This paper also reports new information on its binding affinities of cefoxitin to PBP-1A, 1Bs, 7 and 8. The separation of PBP-7 and 8 recently became possible by an improvement of the electrophoretic technique.

Similar work on another cephamycin antibiotic, CS-1170, has also been carried out and results will be reported elsewhere[3].

Materials and Methods

Assay of Penicillin-binding Activities

A membrane preparation from *E. coli* K-12 strain JE1011 was used in penicillin-binding experiments. The procedures used in isolation of the membrane preparation and its binding of [^{14}C]benzylpenicillin were described in detail previously[2,5]. Experiments on the competition of cefoxitin with [^{14}C]benzylpenicillin for binding the proteins were carried out in reaction mixtures containing in a final volume of 33 μl: 600 μg membrane fraction (as protein), 3 nmol [^{14}C]benzylpenicillin, various amounts of cefoxitin or benzylpenicillin ($0.2\sim125$ mol equivalents to [^{14}C]benzylpenicillin) and 0.05 M sodium

The following abbreviations are used: CFX, cefoxitin; MurNAc, N-acetylmuramic acid; m-A_2pm, meso-diaminopimelic acid; PBP, penicillin-binding protein.

phosphate buffer, pH 7.0. Incubations were carried out at 30°C for 10 minutes and the binding proteins were separated by sodium dodecylsulfate-acrylamide slab-gel electrophoresis and estimated by fluorography as described previously[2,5]. For a good separation of PBP-7 and 8, the slab gel was kept at 10°C during electrophoresis.

Preparation and Assay of Enzymes

For assays of D-alanine carboxypeptidases IA and IB, formation of cross-bridged peptidoglycan and simultaneous release of D-alanine, particulate membrane preparations were prepared from appropriate mutants of *E. coli*. D-Alanine carboxypeptidase IA activity was assayed with enzyme prepared from a *dacB* strain[6], which is defective in D-alanine carboxypeptidase IB activity, while D-alanine carboxypeptidase IB activity was assayed with enzyme from a *dacA* strain[7] which is defective in D-alanine carboxypeptidase IA activity. These activities were assayed by measuring release of D-[^{14}C]alanine from UDP-N-acetylmuramyl(MurNAc)-pentapeptide labeled in D-[^{14}C]Ala-D-[^{14}C]Ala (20 μCi/μmol). Release of D-alanine associated with formation of cross-bridged peptidoglycan was assayed using the enzyme from a double mutant *dacA dacB* strain. Substrate labeled at D-[^{14}C]Ala-D-[^{14}C]Ala (20 μCi/μmol) was used in assays involving release of D-[^{14}C]Ala and substrate labeled at m-[^{14}C]A_2pm (82 μCi/μmol) was used for assay of formation of cross-linked peptidoglycan. The degree of crosslinking in the peptidoglycan labeled at m-[^{14}C]A_2pm that was formed in the reaction was estimated as described previously[5,7]. All enzymatic reactions were carried out at 30°C for 1 hour.

Reagents

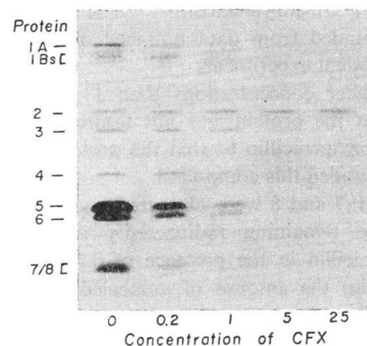
Cefoxitin was obtained from Daiichi Seiyaku Co., Tokyo; potassium benzylpenicillin was a commercial product of Takeda Chemical Industry Co., Osaka, Japan; [^{14}C]benzylpenicillin (potassium 6-phenyl-[1-^{14}C]acetamidopenicillanate, 40~60 μCi/μmol) was a product of the Radiochemical Centre, Ammersham, England. Sodium dodecylsulfate was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium dodecyl-N-sarcosinate (Sarkosyl NL97, Ciba-Geigy) was provided from Kasho Co., Tokyo. Other chemicals used were standard commercial products. Radioactive substrates were prepared as described previously[5,7].

Results and Discussion

Affinities of Cefoxitin to Penicillin-binding Proteins (PBPs) in *E. coli.*

Under recently improved experimental conditions (see Materials and Methods) the PBPs in *E. coli* can be separated by sodium dodecylsulfate/acrylamide gel electrophoresis into nine major proteins, 1A to 8 (Fig. 1). The affinities of cefoxitin to these PBPs were estimated in competition experiment by measuring the binding of [^{14}C]benzylpenicillin to each PBP in the presence of increasing amounts of unlabeled cefoxitin. The fluorogram in Fig. 1 shows that cefoxitin has very high competitive ability with [^{14}C]benzylpenicillin for binding to the low molecular PBPs 7/8, 6, 5, 4, and also the high molecular PBPs 1A and 1Bs. It shows lower competition for PBP-3 and no competition for PBP-2 or 4[1].

Fig. 1. Fluorogram showing competition of cefoxitin with [^{14}C]benzylpenicillin binding in *E. coli*. Concentrations of antibiotics are expressed as molar ratios to [^{14}C]benzylpenicillin.



PBP-1Bs are assumed to be identical with a peptidoglycan-cross-linking, enzyme,[5] and PBP-1A (and/or 2) is thought to function in compensating for lack of PBP-1Bs as a "detour enzyme".[5] PBP-2 and 3 are supposed to be involved in the processes of sphere-rod conversion of cells[8] and of septum formation[8], respectively. PBP-4 is

identical with D-alanine carboxypeptidase IB[6,9] and PBP-5 with D-alanine carboxypeptidase IA[7,10]. PBP-6 seems to be related to PBP-5 but its function is unknown. D-Alanine carboxypeptidases IA and IB, that is PBP-5 and 4, were thought not to be important in the synthesis of peptidoglycan[6,7], but recently it was found that *dacA* mutant cells deficient in D-alanine carboxypeptidase IA are more sensitive to penicillins, cephalosporins and, under certain conditions, cephamycins than *dacA*$^{+}$ cells[10].

PBP-4, which is identical with D-alanine carboxypeptidase IB, may also play some role when the cells are deficient in PBP-1Bs[5]. Nothing is yet known about the functions of PBP-7 and 8.

The facts that cefoxitin has high affinities to PBP-1A, 1Bs and 3 may explain its strong bactericidal potency, because these PBPs are essential in elongation of cell wall peptidoglycan and in septum formation. It is unknown whether the high affinities to PBP-4 and 5 (and 6) contribute to the antibacterial activity of the antibiotic, because these PBPs do not seem to be very important for cell proliferation under normal conditions. The recent finding[10] that a defect in PBP-5 results in super-sensitivity of the cells to several antibiotics suggests, that binding of an antibiotic to this PBP has a secondary effect of increasing the sensitivity of the cells to the antibiotic.

Table 1 shows the affinities of cefoxitin to each *E. coli* PBP, calculated as the concentration ratios of cefoxitin to [^{14}C]benzylpenicillin needed for 50% competition, *i. e.*, 50% inhibition of binding of [^{14}C]benzylpenicillin. Table 1 also shows the concentration ratios for 50% competition of unlabeled benzylpenicillin with [^{14}C]benzylpenicillin. Theoretically, the values for homologous competition should be 1 for each PBP, but in practice they vary from 0.8 to 2.5 for some unknown reason.

Table 1. Competition of antibiotics with [^{14}C]benzylpenicillin for binding to PBPs in cytoplasmic membranes of *E. coli in vitro*.

PBP	Concentration[a] of	
	Cefoxitin	Benzylpenicillin
1A	0.3	0.8
1Bs	0.8	0.8
2	>25	0.8
3	1.5	0.9
4	0.6	0.8
4′	>25	1
5	0.3	2.5[b]
6	1.1	2.2[b]
7/8[c]	< 0.2[d]	0.9

[a] Concentration as a molar ratio to [^{14}C]benzylpenicillin required for 50% inhibition of binding of [^{14}C]benzylpenicillin. The table is compounded from data obtained in several independent experiments.

[b] Higher concentrations than 1 indicate either that the protein was not saturated with [^{14}C]benzylpenicillin or that the protein released or degraded this compound.

[c] PBP-7 and 8 were measured together.

[d] The remaining radioactivity of [^{14}C]benzylpenicillin in the presence of 0.2 times (molar ratio) the amount of unlabeled cefoxitin was 25%.

Of the enzyme reactions with which the PBPs can be correlated, only 4 reactions can now be measured: the D-alanine carboxypeptidase IA reaction, for which PBP-5 is responsible, the D-alanine carboxypeptidase IB reaction, for which PBP-4 is responsible, formation of cross-linked peptidoglycan and concomitant release of D-alanine. PBP-1Bs seems to be responsible for the last 2 reactions, but it is possible that the release of D-alanine concomitant with the cross-linking reaction is due to another enzyme(s). The enzymatic reactions due to PBP-1A, 2, 3, 6, 7 and 8 have not yet been identified. Table 2 shows the concentrations of cefoxitin causing 50% inhibition of these 4 reactions. Cefoxitin inhibited all 4 reactions at much lower concentrations than benzylpenicillin. In these 4 reactions the inhibitory potencies of the antibiotic correlate approximately with its affinities to the corresponding PBPs.

Table 2. Concentrations of antibiotics required for 50% inhibition of the activities of D-alanine carboxypeptidases IA and IB, the peptidoglycan cross-linking reaction and concomitant release of D-alanine

Exp.	Reaction	PBP responsible for the reaction	Concentration (μg/ml) of	
			Cefoxitin	Benzylpenicillin
1	D-Alanine carboxypeptidase IA	5	0.02	1
2	D-Alanine carboxypeptidase IB	4	0.005	0.01
3	Cross-linking of peptidoglycan *in vitro*	1Bs	0.5	3
4	D-Alanine release concomitant with cross-linking of peptidoglycan *in vitro*	(1Bs?)	0.6	3

Reaction mixtures contained in a final volume of 33 μl: exps. 1 and 2, 2 μmol Tris-HCl buffer, pH 8.6, 1 μmol $MgCl_2$, 0.44 nmol UDP-MurNAc-L-Ala-D-Glu-m-A_2pm-D-[^{14}C]Ala-D-[^{14}C]Ala, 50 nmol 2-mercaptoethanol, 0.1% (wt/vol, final) Triton X-100 and 100 μg enzyme (as protein); exp. 3, 2 μmol Tris-HCl buffer, pH 8.6, 1 μmol $MgCl_2$, 0.35 nmol UDP-MurNAc-L-Ala-D-Glu-m-[^{14}C]A_2pm-D-Ala-D-Ala, 10 nmol UDP-GlcNAc, 50 nmol 2-mercaptoethanol and 100 μg enzyme (as protein); exp. 4, as for exps. 1 and 2, except that 10 nmol UDP-GlcNAc was present and 0.1% (wt/vol, final) Triton X-100 was omitted.

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