BINDING OF BICYCLOMYCIN TO INNER MEMBRANE PROTEINS OF *E. COLI*

AKIRA SOMEYA, MANABU ISEKI* and NOBUO TANAKA

Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
*Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan

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[^{14}C]Bicyclomycin was observed to bind to several inner membrane proteins of *E. coli*, but not to outer membrane proteins. In SDS polyacrylamide (12.5%) slab gel electrophoresis, 4 major bands and 3 minor bands of binding proteins were demonstrated. Benzylpenicillin showed no competition with bicyclomycin for bicyclomycin-binding proteins (BBPs), and bicyclomycin no competition with penicillin for penicillin-binding proteins (PBPs). The absence of competition and the difference of mobilities of BBPs and PBPs suggested that BBPs differ from PBPs. The molecular weights of BBPs were estimated in comparison with PBPs on slab gel electrophoresis: BBP-1 *ca.* 93,000, BBP-2 72,000, BBP-3 53,000, BBP-4 46,000, BBP-5 41,000, BBP-6 30,000, and BBP-7 27,000. The binding to all the proteins seemed to be irreversible in that the antibiotic was not released from the proteins during a 3-hour incubation. From the kinetics of binding it is likely that the binding is a simple bimolecular irreversible reaction. At saturation, 8 pmoles of [^{14}C]bicyclomycin were bound to 1 mg (dry weight) of *E. coli*, *i.e.* 2,400 molecules per cell. An estimate of the number of molecules of each BBP per cell was calculated from measurements of the amount of bicyclomycin bound per cell and the relative proportions of the antibiotic bound to each protein.

The results indicated that, besides PBPs, there exist(s) inner membrane protein(s) participating in cell division.

Bicyclomycin, obtained from the culture broth of *Streptomyces sapporoensis*, is a cyclic peptide antibiotic of an unique structure, inhibiting growth of certain Gram-negative bacteria[1~4]. The mechanism of action of the antibiotic has been studied with *Escherichia coli*[5]. Bicyclomycin causes the formation of multinucleate and aseptate filaments as well as osmotically fragile spheroplast-like bodies. Protein and nucleic acid syntheses are not significantly affected by the antibiotic *in vivo* or *in vitro*. Bicyclomycin has been observed to inhibit biosynthesis of the bound form of lipoprotein, which exists in the outer membrane and is covalently linked to peptidoglycan[5].

On the other hand, the β-lactam group of antibiotics (penicillins and cephalosporins) has been reported to bind irreversibly to bacterial membranes and multiple penicillin-binding proteins (PBPs) have been detected in the membranes of various bacteria (cf. a review[6]). Among them, PBP–3, is considered to participate in cell division[7,8]. However, the process of cell division may be more complex and may also involve other proteins.

[^{14}C]Bicyclomycin has been prepared by a biosynthetic procedure, and its binding to the envelope of *E. coli* has been studied in comparison with the binding of penicillin to PBPs. The results are presented in this publication.

Materials and Methods

[^{14}C]Benzylpenicillin (53 Ci/mole) was purchased from Radiochemical Centre, Amersham, England, benzylpenicillin from Meiji Seika Co., Tokyo, and Sarkosyl NL97 (sodium-lauryl sarcosinate, Ciba-Geigy) from Kasho, Co., Tokyo.

E. coli JE5505 (lpo⁻) and JE5506 (lpo⁺) were generously given by Dr. Y. HIROTA, National Institute of Genetics, Mishima, Shizuoka-ken, Japan[15]. *E. coli* ATCC 27166, a mutant hypersensitive to bicyclomycin[5], and *B. subtilis* ATCC 6633 were used. Cells were grown in LENNOX broth[9], and were harvested in late exponential phase of growth.

Preparation of [^{14}C]bicyclomycin:

[^{14}C]Bicyclomycin was biosynthesized by introducing L-[^{14}C]leucine and L-[^{14}C]isoleucine into the culture broth of *Streptomyces sapporoensis*. It was extracted following the procedure as described previously[1], and was purified by thin-layer chromatography, using a solvent system ($CHCl_3$ - MeOH, 5: 1)[4]. The specific activity was 8.0 Ci/mole, and the radiochemical purity more than 96%.

Binding of [^{14}C]bicyclomycin to the cell envelope:

Washed cell envelope, consisting of inner (cytoplasmic) and outer membranes, and peptidoglycan, was prepared according to the procedure of SPRATT[8]. Bicyclomycin-binding proteins were detected by binding of [^{14}C]bicyclomycin (final concentration 250~350 μg/ml) to the cell envelope (about 20 mg protein/ml 10 mM sodium phosphate buffer, pH 7.0) for 30 minutes at 30°C. The reaction was terminated by addition of non-radioactive bicyclomycin (final concentration 35 mg/ml) and Sarkosyl NL97 [final concentration 1% (w/v)]. After solubilization of the inner membrane for 20 minutes at room temperature, the Sarkosyl-insoluble fraction (outer membrane and peptidoglycan) was removed by centrifugation at 100,000×g for 40 minutes at 0~4°C. Then the extract was fractionated on a SDS polyacrylamide (12.5%) slab gel electrophoresis system[10]. Penicillin-binding proteins were comparatively observed by a similar method, using [^{14}C]benzylpenicillin, as described by SPRATT[8]. The gels were dried and fluorography was carried out on pre-fogged Kodak RP Royal X-ray film[11]. The exposure period for fluorography was about 5 weeks at −75°C. Apparent molecular weights of bicyclomycin-binding proteins were measured by comparison of their mobilities on SDS polyacrylamide slab gels with those of 8 known penicillin-binding proteins[8,14].

Release of [^{14}C]bicyclomycin bound to the inner membrane:

To determine spontaneous release of bound bicyclomycin, the cell envelope was labelled with [^{14}C]bicyclomycin for 30 minutes at 30°C as described above. A 100-fold excess of cold bicyclomycin was added to the mixture, from which samples of 400 μl were removed immediately and at intervals during continued incubation at 30°C, and poured into tubes containing 20 μl of 20% (w/v) Sarkosyl in an ice bath. The Sarkosyl-soluble proteins were fractionated on SDS polyacrylamide slab gel electrophoresis, and fluorography was done as described above. Quantative analysis of bound [^{14}C] bicyclomycin was carried out by examining the X-ray films in a micro-densitometer[11].

Binding of [^{14}C]bicyclomycin to whole cells of bacteria:

Various concentrations of [^{14}C]bicyclomycin were incubated with bacterial cell suspensions (8~11 mg dry weight/ml 50 mM sodium phosphate buffer, pH 7.0) for 10 minutes at 30°C. The binding of the [^{14}C]antibiotic was terminated by addition of excess cold bicyclomycin, and examined by centrifugation[12] or trichloroacetic acid (5%) precipitation[13].

Results

Localization of Bicyclomycin-binding Proteins in the Cell Envelope of *E. coli*

[^{14}C]Bicyclomycin-binding proteins (BBPs) were found in the Sarkosyl-soluble fraction of *E. coli* envelope, but not in the Sarkosyl-insoluble fraction. The former was reported to consist of the inner membrane and the latter of the outer membrane[17]. In SDS polyacrylamide (12.5%) slab gel electrophoresis, 4 major bands of BBPs and 3 minor bands were observed (Fig. 1). The results suggested that bicyclomycin may bind to the inner membrane but not to the outer membrane.

Competition of Benzylpenicillin with [^{14}C]Bicyclomycin for Bicyclomycin-binding Proteins

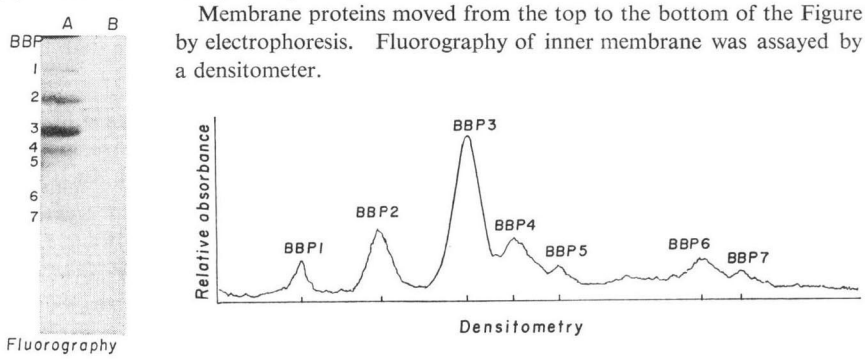
Fig. 1. Detection of *E. coli* [^{14}C]bicyclomycin-binding proteins (BBPs) and their location in the inner membrane.

[^{14}C]Bicyclomycin was incubated with *E. coli* cell envelope fraction for 30 minutes at 30°C. The 1% Sarkosyl-soluble (inner membrane) and -insoluble (outer membrane) fractions were obtained, and were fractionated on an SDS polyacrylamide (12.5%) slab gel electrophoresis as described in "Materials and Methods".

(A) Inner membrane (Sarkosyl-soluble fraction)

(B) Outer membrane (Sarkosyl-insoluble fraction)

Membrane proteins moved from the top to the bottom of the Figure by electrophoresis. Fluorography of inner membrane was assayed by a densitometer.



For the purpose of determining whether binding proteins or sites of bicyclomycin are identical with those of penicillin, the envelope fraction was pretreated with benzylpenicillin prior to the binding of [^{14}C]bicyclomycin, as described in "Materials and Methods". The binding of penicillin to inner membrane proteins at saturation level did not block the subsequent binding of [^{14}C]bicyclomycin to the membrane proteins (BBPs) (Fig. 2 A and B). The results suggested that the binding sites of bicyclomycin may be different from those of penicillin.

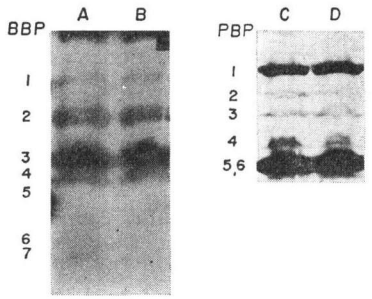
Fig. 2. Competition of penicillin with [^{14}C]bicyclomycin for bicyclomycin-binding proteins (BBPs) and competition of bicyclomycin with [^{14}C]benzylpenicillin for penicillin-binding proteins (PBPs).

(A) The envelope fraction was incubated with 250 μg/ml of [^{14}C]bicyclomycin for 30 minutes at 30°C.

(B) The envelope fraction was preincubated with 1 mg/ml of cold benzylpenicillin for 10 minutes at 30°C, and then treated as (A).

(C) The envelope was incubated with 31 μg/ml of [^{14}C]benzylpenicillin for 10 minutes at 30°C.

(D) The envelope was preincubated with 1 mg/ml of cold bicyclomycin for 30 minutes at 30°C, and then treated as (C).



Competition of Bicyclomycin with [^{14}C] Benzylpenicillin for Penicillin-binding Proteins

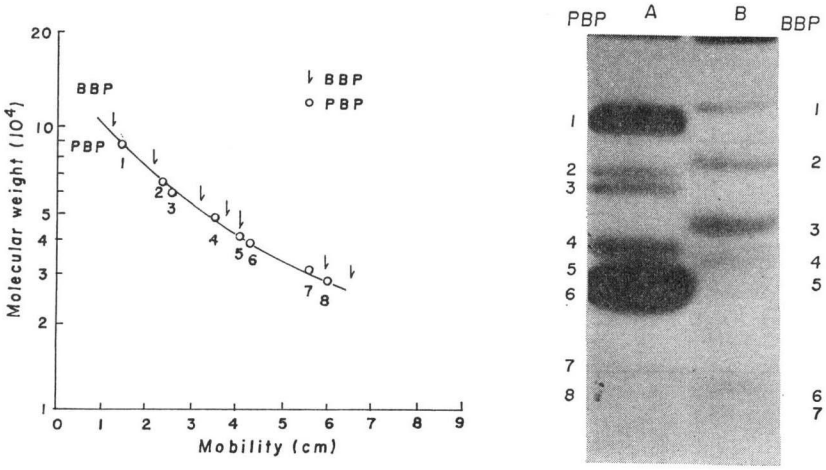
For the same purpose, the envelope was treated with bicyclomycin prior to the binding of [^{14}C]benzylpenicillin. The binding of bicyclomycin did not significantly interfere with the subsequent binding of [^{14}C]penicillin to PBPs (Fig. 2 C and D). The mobilities of BBPs were observed to be different from those of PBPs on a single slab gel electrophoresis (Fig. 3). The results suggested that the binding proteins of penicillin may be different from those of bicyclomycin.

Estimation of Molecular Weights of Bicyclomycin-binding Membrane Proteins

The molecular sizes of BBPs (bicyclomycin-binding proteins) were estimated by comparing the

Fig. 3. Estimation of molecular weights of bicyclomycin-binding proteins (BBPs).

The molecular weights of BBPs (B) were estimated by comparison of mobilities on an SDS polyacrylamide (12.5%) slab gel electrophoresis with those of PBPs (A). The molecular weights of PBPs 1, 2, 3, 4, 5, 6, 7 and 8 were reported to be 91,000, 66,000, 60,000, 49,000, 42,000, 40,000, 32,000 and 29,000[8,14].



mobilities on SDS polyacrylamide (12.5%) slab gel electrophoresis with those of 8 PBPs (penicillin-binding proteins) of known molecular weights[8] (Fig. 3 A). The molecular weights of major 4 BBPs were estimated as *ca.* 93,000, 72,000, 53,000, and 46,000, as well as minor 3 BBPs as *ca.* 41,000, 30,000, and 27,000 by the procedure of WEBER and OSBORN[14] (Fig. 3). The proteins were named BBPs (bicyclomycin-binding proteins) 1, 2, 3, 4, 5, 6 and 7, according to the size of molecular weights (Fig. 3 B).

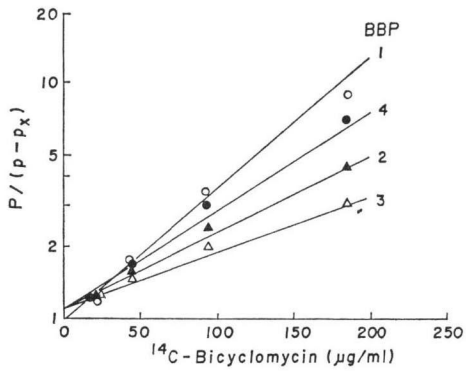
Kinetics of Binding of [^{14}C]Bicyclomycin

[^{14}C]Bicyclomycin was incubated with aliquots of the envelope fraction for 30 minutes at 30°C. The antibiotic concentrations were 23, 46, 92 and 184 μg/ml, and the extent of binding of each protein was measured by the procedure described in "Materials and Methods". The binding of the [^{14}C]antibiotic to BBPs 1~4 was treated as a simple bimolecular irreversible reaction. For such a reaction the degree of binding of bicyclomycin must be proportional to the product of the antibiotic concentration and the incubation time. As illustrated in Fig. 4, BBPs 1~4 appeared to bind to bicyclomycin in accord with the above assumption of kinetics. The results suggested that the binding of the antibiotic to BBPs may be a bimolecular irreversible reaction.

Fig. 4. Kinetics of binding of [^{14}C]bicyclomycin to membrane proteins.

BBP 1, 2, 3 and 4 represents bicyclomycin-binding protein 1, 2, 3 and 4.

P is saturation level of bicyclomycin binding, and Px is each level of binding at the antibiotic concentration x (μg/ml).



Release of [^{14}C]Bicyclomycin Bound to Membrane Proteins

The release of bound [^{14}C]bicyclomycin from each BBP was measured during 3 hours of incubation

Fig. 5. Release of [^{14}C]bicyclomycin bound to the inner membrane.

BBP 1, 2, 3 and 4 represents bicyclomycin-binding protein 1, 2, 3 and 4.

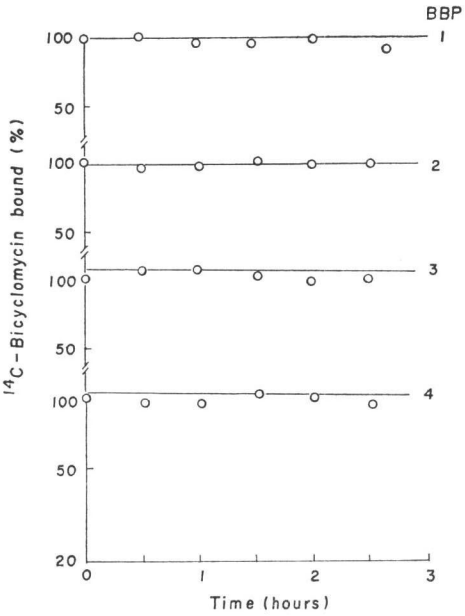
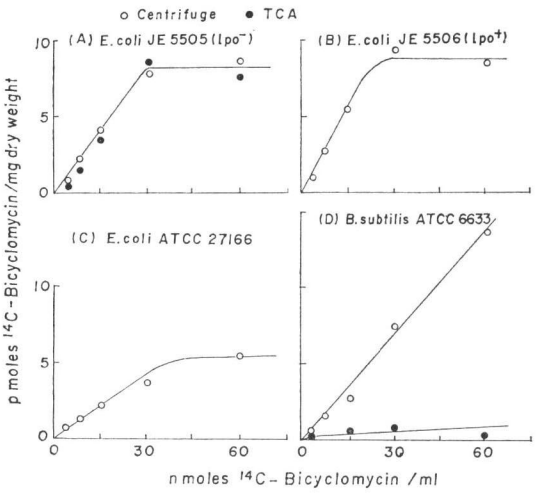


Fig. 6. Binding of [^{14}C]bicyclomycin to whole cells of bacteria.

The binding was observed by centrifugation (○), or TCA precipitation (●).



at 30°C. As shown in Fig. 5, no significant release of [^{14}C]bicyclomycin from BBPs 1~4 was observed. Other minor BBPs also exhibited no significant release of the antibiotic during 3 hours (data are not shown). The results suggested that no enzyme which can cleave the bond(s) between the antibiotic and BBPs was present in the envelope. It seems to be consistent with the observation that bicyclomycin was resistant to bacterial degradation[3].

Binding of [^{14}C]Bicyclomycin to Whole Cells of Bacteria

[^{14}C]Bicyclomycin was observed to irreversibly bind to *E. coli* cells (Fig. 6 A, B and C). Approximately 8 pmoles of bicyclomycin bound to 1 mg (dry weight) of *E. coli* JE5505 and JE5506 at saturation. In the case of *E. coli* ATCC 27166, a mutant hypersensitive to bicyclomycin, the amount of bound [^{14}C]bicyclomycin was less than those of *E. coli* JE5505 and JE5506. Bound bicyclomycin was not released by dilution in buffers, incubation with unlabelled bicyclomycin, or extraction with 5% TCA (trichloroacetic acid).

On the contrary, [^{14}C]bicyclomycin appeared to bind to the cells of *Bacillus subtilis* ATCC 6633 as demonstrated by the centrifugation method; but the bound antibiotic was released by extraction with 5% TCA (Fig. 6 D).

Estimation of Relative Amounts of Bicyclomycin-binding Proteins (BBPs) per Cell

The results, presented in Fig. 6 A and B, indicated that on average *ca.* 2,400 molecules of bicyclomycin were bound per cell at saturation, provided that 2×10^9 growing cells were contained in 1 mg (dry weight) of *E. coli*. Relative amounts of bound [^{14}C]bicyclomycin were estimated by assaying density of bands on a X-ray film, using a micro-densitometer (Fig. 1). As presented in Table 1, BBP-1 was observed to bind with 9% of total bound bicyclomycin, BBP-2 19%, BBP-3 55%, BBP-4 7%, and

BBPs-5, -6 and -7 10%. The number of molecules of each protein (BBP) per cell was estimated as shown in Table 1 from the molecules of bound bicyclomycin per cell and relative proportions of the antibiotic bound to each BBP, assuming a stoichiometry of 1 mole bicyclomycin binding to 1 mole BBP. It was found by the method employed that a single cell contained on average 220 molecules of BBP-1, 450 of BBP-2, 1,320 of BBP-3, 170 of BBP-4, and 240 of BBPs-5, -6 and -7. These values might vary with growth conditions and strains used.

Table 1. Estimation of molecular weights and relative abundance of bicyclomycin-binding proteins (BBPs).

BBP (protein)	Molecular weight	Binding of bicyclomycin (% of total)	Molecules per cell
1	93,000	9	220
2	72,000	19	450
3	53,000	55	1,320
4	46,000	7	170
5	41,000		
6	30,000	10	240
7	27,000		

Discussion

Bicyclomycin has been reported to inhibit biosynthesis of murein-lipoprotein, particularly that of the bound form[5]. On the other hand, an *E. coli* mutant JE5505 (lpo$^-$), lacking murein-lipoprotein, has been recently isolated and this mutant grows well under a wide range of growth conditions[15], indicating that lack of murein-lipoprotein may not be fatal to *E. coli*. Bicyclomycin has been found to inhibit growth of *E. coli* JE5505 (lpo$^-$) at the same level as for the parent strain JE5506 (lpo$^+$) (data are not shown). The results suggest that inhibition of murein-lipoprotein biosynthesis by bicyclomycin may not be the primary action but rather a secondary action.

The observation that bicyclomycin makes *E. coli* filamentous[5], indicates a possibility of inhibition of peptidoglycan biosynthesis. However, bicyclomycin has been found not to prevent the incorporation of diaminopimelic acid or alanine into peptidoglycan *in vivo*. The mechanism of action of bicyclomycin seems to be different from that of penicillin. It is also supported by the facts that bicyclomycin does not possess a β-lactam ring in the molecule and BBPs are different from PBPs.

SPRATT[7] has indicated that PBP-3 is involved in cell division or septum formation. Bicyclomycin has been observed to block cell division without interacting with PBP-3. The results suggest that cell division may involve more complex mechanism and there exist(s) inner membrane protein(s), participating in cell division, besides PBP-3.

As demonstrated by the TCA precipitation method, [^{14}C]bicyclomycin binds to *E. coli* but not to *B. subtilis* (Fig. 6). The results seem to be in accord with the antimicrobial spectrum: *i.e.* the antibiotic inhibits growth of *E. coli* but not that of *B. subtilis*[1,3].

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