Biochem. J. (1993) 291, 19–21 (Printed in Great Britain)

RESEARCH COMMUNICATION

A new, highly sensitive method for the detection and quantification of penicillin-binding proteins

Moreno GALLENI, Bernard LAKAYE, Sophie LEPAGE, Marc JAMIN, Iris THAMM, Bernard JORIS and Jean-Marie FRÈRE*

Laboratoire d'Enzymologie and Centre d'Ingéniérie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart-Tilman, Belgium

A new method for the identification and quantification of penicillin-binding proteins is described which uses fluorescein-coupled penicillins. It allows the rapid detection of 0.2 pmol with the naked eye and 2 fmol with the help of an A.L.F. automatic DNA sequencer. Direct labelling can also be performed on whole bacterial cells.

INTRODUCTION

The cytoplasmic membranes of all eubacteria contain several penicillin-binding proteins (PBPs) (Spratt and Pardee, 1975). Inactivation of one or more of these proteins by β-lactam antibiotics results in cell death, and a large number of studies have been devoted to their detection (Spratt, 1977), to the establishment of their physiological roles and to the analysis of their interactions with penicillins, cephalosporins and related compounds (Frère and Joris, 1985; Spratt and Croomie, 1988; Ghuysen, 1991). Routinely, PBPs are detected by incubating membrane preparations with radiolabelled penicillins, separating the proteins by SDS/PAGE and revealing the PBPs by fluorography. This time-consuming procedure can take several days before the results can be analysed. We here describe a new, rapid and sensitive method for the detection of PBPs which does not involve the utilization of radiolabelled compounds.

MATERIALS AND METHODS

Chemicals and strains

N-Hydroxysuccinimide-activated fluorescein (Flu) was from Boehringer-Mannheim (Mannheim, Germany). 6-Aminopenicillanic acid (6APA) was given by SmithKline Beecham (Heppignies, Belgium). The *Escherichia coli* strain was HB101 (Boyer and Roulland-Doussoix, 1969). The *Bacillus licheniformis* 749/P22 strain exhibiting a very low β-lactamase activity was given by Dr. D. A. Dubnau (Dubnau and Pollock 1965).

Methods

N-Hydroxysuccinimide-activated fluorescein was coupled to 6APA or glycyl-6APA, yielding respectively Flu-6APA and Flu-glycyl-6APA. Typically, to 10 mg of 6APA dissolved in 10 ml of 10 mM sodium phosphate buffer, pH 7, was added an equimolar quantity of *N*-hydroxysuccinimide-activated fluorescein diluted in the same buffer. The mixture was left overnight at 20 °C and the product purified by chromatography on a C_{18} MN reverse-phase column (Macherey–Nagel, Darmstadt, Germany). The yield was about 20%. The detailed syntheses will be described elsewhere.

For direct visual detection and quantification, the fluorescent compounds were first made to react with purified soluble PBPs. Various quantities of the *Actinomadura* R39 DD-peptidase (13.2–0.2 pmol) were incubated with 8.3 μM Flu-glycyl-6APA for 30 min at 37 °C in a total volume of 100 μl of 10 mM Tris/HCl buffer, pH 7.5. Denaturation buffer was added (0.1 M Tris/HCl, pH 6.8, containing 25% glycerol, 2% SDS, 20% β-mercaptoethanol and 0.02% Bromophenol Blue) and the sample was heated at 100 °C for 1 min. Samples were then loaded on a 12%-acrylamide/SDS gel (9 cm × 7 cm) and the electrophoresis was performed for 45 min at 200 V (15 mA). Detection and quantification were made under u.v. light with the help of a two-dimensional densitometer (Cybertech CS-1; Dalton, Waalwijk, The Netherlands).

Electrophoresis of the labelled *Actinomadura* R39 DD-peptidase (35–2 fmol) was also performed on a 12%-polyacrylamide/SDS gel in an A.L.F. DNA sequencer (Ansorge et al., 1987). Detection of the labelled protein was done by a laser beam, which increased the sensitivity more than 100-fold. The laser beam was 9 cm from the sample slots. To each sample, an identical amount (10 fmol) of the *Streptomyces* R61 DD-peptidase, previously labelled with Flu-glycyl-6APA was added, thus providing an internal standard. The ratios of the areas under the peaks corresponding to the labelled *Actinomadura* R39 DD-peptidase (M_r 53000) and the *Streptomyces* R61 DD-peptidase (M_r 37500) enzymes (relative intensities) were calculated.

The labelling of *E. coli* HB101 PBPs was carried out in isolated membranes or in permeabilized whole cells. The membranes were prepared as described by Spratt (1977) and suspended in 50 mM sodium phosphate buffer, pH 7.0, at an estimated concentration of 20 mg/ml. Samples (10 μl) of the membrane preparation were incubated with 10 μM Flu-glycyl-6APA for 30 min at 30 °C. For competition assays, the membrane were first treated with unlabelled antibiotics (aztreonam, mecillinam and imipenem) at a final concentration of 10 μM during 10 min at 30 °C. After incubation with the fluorescent penicillin, the membranes were washed with phosphate buffer, dissolved in 10 μl of denaturation buffer and heated at 100 °C for 2 min. The samples were then diluted 60-fold with the denaturation buffer, and 10 μl was loaded on top of a 7.5% polyacrylamide gel containing 0.1% SDS. Labelling of whole *E. coli* cells was performed on 2 ml samples withdrawn at an A_{550} of 1.0. The cells were isolated by centrifugation, suspended in 50 μl of 50 mM sodium phosphate buffer, pH 7.0, and permeabilized by three cycles of freezing and thawing. Flu-glycyl-6APA was then added

Abbreviations used: PBP, penicillin-binding protein; Flu, *N*-hydroxysuccinimide-activated fluorescein; 6APA, 6-aminopenicillanic acid.

* To whom correspondence should be sent.

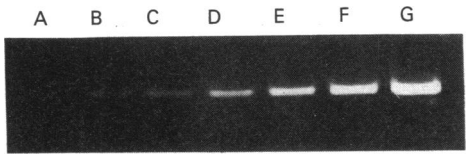

Figure 1 Labelling and detection of the *Actinomadura* R39 DD-peptidase

Various quantities of the *Actinomadura* R39 DD-peptidase were loaded on an SDS/12%-polyacrylamide gel. Lanes: A, 0.22 pmol; B, 0.44 pmol; C, 0.88 pmol; D, 1.65 pmol; E, 3.3 pmol; F, 6.6 pmol; G, 13.2 pmol.

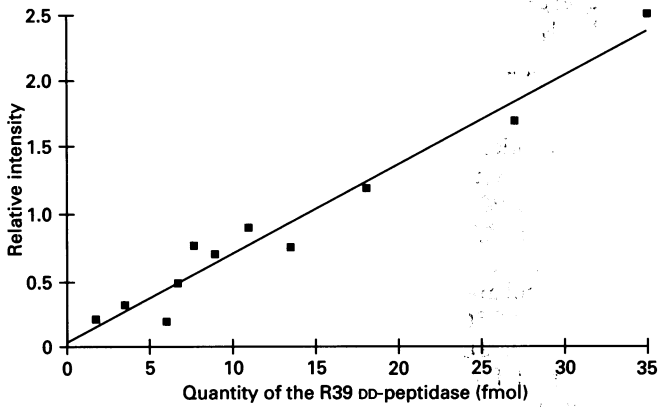

Figure 2 Quantification of the labelled *Actinomadura* R39 DD-peptidase performed with the help of the A.L.F. DNA sequencer

Various quantities of the *Actinomadura* R39 DD-peptidase were loaded on an SDS/12%-polyacrylamide gel. The graph shows the ratios of the areas under the peaks corresponding to the labelled *Actinomadura* R39 DD-peptidase (M_r 53000) and the *Streptomyces* R61 DD-peptidase (M_r 37500) enzymes (relative intensity) versus the quantities of the *Actinomadura* R39 DD-peptidase. The labelled DD-peptidase of *Streptomyces* R61 was used as an internal standard.

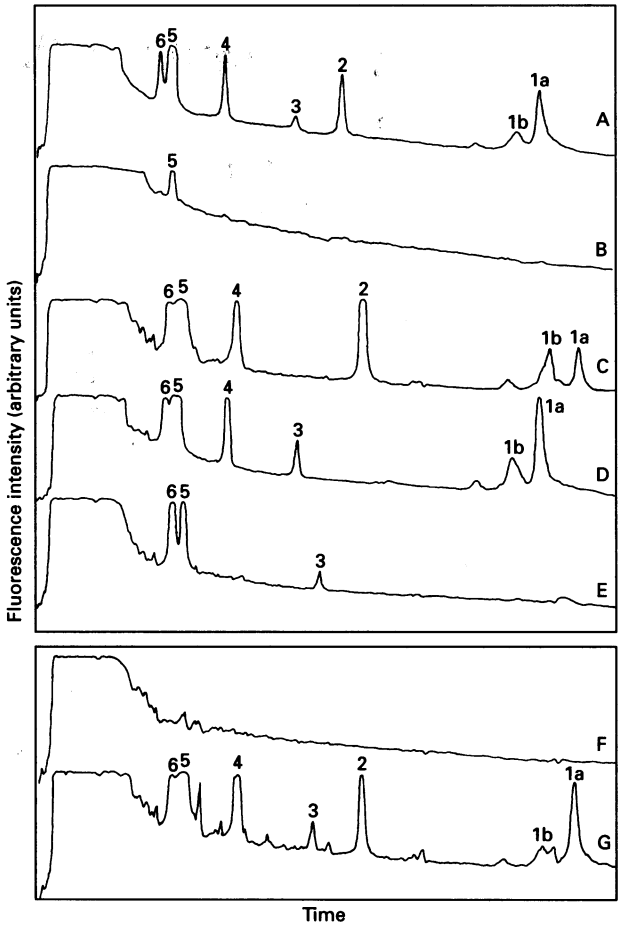

Figure 3 Detection of the *E. coli* HB101 PBPs in isolated membranes (A–E) and in permeabilized whole cells (F, G)

Lane (**A**) represents the pattern obtained with the membrane sample treated with Flu-glycyl-6APA. Samples B–E were first treated with unlabelled antibiotics at final concentrations of 10 μM for 10 min at the same temperature and then incubated with Flu-glycyl-6APA. Benzylpenicillin (**B**) completely inhibited the labelling of all PBPs. In agreement with their described specificities, aztreonam (specific for PBP3) (**C**), mecillinam (specific for PBP2) (**D**) and imipenem (**E**) reacted with PBPs 2, 3 and 4, 1b and 1a (Frère and Joris, 1985) respectively. Lane G represents the labelling of the *E. coli* PBPs in whole cells; 10 μl of the preparation was loaded on the gel. Lane F represents the same sample preincubated with 200 μM benzylpenicillin before incubation with Flu-glycyl-6APA. PBP peaks are numbered. PBP 1a was detected after a 4 h run.

at a final concentration of 10 μM, and the mixture was incubated at 30 °C for 30 min. The cells were isolated by centrifugation and suspended in 50 μl of denaturing buffer, to which 5 μl of β-mercaptoethanol was added, and the mixture was heated at 100 °C for 2 min. Then 10 μl of the solution was deposited on the gel.

The PBPs from *Bacillus licheniformis* were also detected. Membranes were prepared as described by Chase et al. (1978) and suspended in 40 mM sodium phosphate buffer, pH 7, at a final concentration of 9 mg/ml. Samples (10 μl) were first treated with 5 μM β-iodopenicillanic acid for 5 min at 37 °C to inactivate any residual β-lactamase activity, added with 5 μl of 3 μM Flu-glycyl-6APA, and incubated for 15 min at 37 °C. The membranes were then isolated by centrifugation, washed with the phosphate buffer, suspended in 7.5 μl of denaturing buffer and heated at 100 °C for 2 min. The samples were diluted 14-fold, and 10 μl was deposited on the gel.

Labelling of whole cells was performed on 0.5 ml samples withdrawn at an A_{550} of 1.0. After centrifugation, the cells were suspended in 70 μl of 40 mM phosphate buffer, incubated for 20 min at 37 °C with 1 μM Flu-glycyl-6APA, separated by centrifugation, washed with the phosphate buffer and suspended in 10 μl of denaturing buffer. The samples were heated for 3 min at 100 °C, diluted 10-fold, and 10 μl samples were deposited on top of a 10%-polyacrylamide gel containing 0.1% SDS.

RESULTS AND DISCUSSION

Figure 1 displays a photograph of a minigel after migration of samples containing increasing quantities of the *Actinomadura* R39 DD-peptidase. It shows that 0.2 pmol could be detected under u.v. light with the naked eye.

With the help of an automatic DNA sequencer, we were able to observe 2 fmol of the same DD-peptidase. Figure 2 shows a calibration curve obtained after labelling of this enzyme by Flu-glycyl-6APA, demonstrating that quantification might become difficult below 7 fmol, but quantities as low as 2 fmol were reproducibly detected.

The method was subsequently applied to membrane preparations and whole cells. Figure 3 shows the results obtained with *E. coli* PBPs as detected by the laser beam. The high sensitivity of the method should be emphasized, since the recordings were obtained with 3 μg of total membrane proteins. Note that the

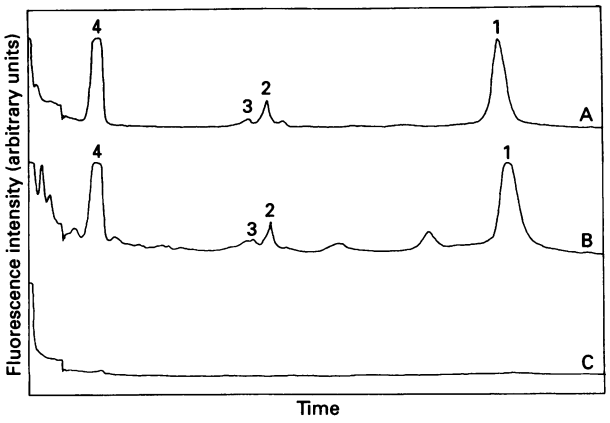

Figure 4 Detection of the *B. licheniformis* PBPs

Lane (**A**) represents 10 μl of a 14-fold-diluted *B. licheniformis* membrane sample treated with Flu-glycyl-6APA, and lane (**C**) the effects of a 10 min preincubation with 10 μM benzylpenicillin. Lane (**B**) represents the PBPs pattern obtained when whole cells were labelled. PBP 1 was detected after a 5 h run.

detection of the same proteins labelled with radioactive penicillins requires about 100 μg of total membrane proteins. Figure 3 also shows results obtained after the labelling of whole permeabilized cells where all the usual PBPs could be observed in addition to some unidentified proteins. Interestingly, even these proteins were no longer labelled if the cells had been preincubated with benzylpenicillin. In agreement with their described specificities, aztreonam, mecillinam and imipenem respectively reacted with PBPs 3, 2 and 4, 1b and 1a. Pre-treatment of the sample with unlabelled benzylpenicillin completely abolished the fixation of the fluorescent penicillin. This indicated that the fluorescent probe and the unlabelled penicillin reacted with the same side chain, i.e. the active-site serine residue. The largest PBP (PBP 1a) was detected after a 4 h run. Finally, the PBPs of *B. licheniformis* (PBP 1, 2, 3 and 4) were also analysed by the new method (Figure 4).

In conclusion, the utilization of fluorescein-labelled penicillins supplies a technique for studying PBPs which is more rapid and sensitive than the classical fluorographic methods. Indeed, detection of 0.2 pmol of [^{14}C]benzylpenicillin-labelled DD-peptidase of *Streptomyces* R61 (55 mCi/mmol) requires a 10-day exposure, whereas that of the *E. coli* PBPs with the same but ^{3}H-labelled (5000 mCi/mmol) compound takes about 1 week. The *E. coli* PBPs can be more rapidly detected (12–48 h) with ^{125}I-labelled derivatives of ampicillin (Schwarz et al., 1981) or *p*-hydroxybenzylpenicillin (Masson and Labia, 1983), but, with this short-lived radioisotope, new samples of labelled antibiotic must be prepared frequently. This contrasts with the normal stability of the fluorescent derivatives, which in addition also permit an easy quantification of the proportions of the different PBPs, and consequently can routinely be used for detailed kinetic studies of the interaction between β-lactams and PBPs.

In the present study, the kinetic parameters characterizing the rates of acylation of the various membrane PBPs were not determined, and we cannot be absolutely certain that they were all 100 % labelled. However, the proportions of PBPs 1A + 1B, 2 and 3 in the *E. coli* membranes found here (100, 35 and 20 respectively) are in agreement with those reported (100, 10, 20) by Spratt (1977). The method can also be utilized to estimate PBP concentrations during their production and purification. Indeed, the electrophoretic separation can be rapidly performed (45 min) and small quantities can be immediately detected with the naked eye.

This work was supported in part by the Belgian government in the frame of the 'Pôles d'attraction interuniversitaires' (PAI no. 19), an 'Action concertée' with the Belgian government (convention 89/94-130), the 'Fonds de la recherche scientifique médicale' (contrat no. 3.4537.130) and a 'convention tripartite' between the Région wallonne, SmithKline Beecham, U.K., and the University of Liège. M.G. and B.J. are respectively 'Chargé de recherche' and 'Chercheur qualifié' of the FNRS (Brussels, Belgium), and M.J. is a Research fellow of the IRSIA (Brussels, Belgium). We thank D. Raze, A. Brans and A. Labye for their help.

REFERENCES

Ansorge, W., Sproat, B., Stegemann, J., Schawger, C. and Zenke, M. (1987) Nucleic Acids Res. **15**, 4593–4602
Boyer, H. W. and Roulland-Doussoix, D. (1969) J. Mol. Biol. **41**, 459–472
Chase, H. A., Reynolds, P. E. and Ward, J. B. (1978) Eur. J. Biochem. **88**, 275–285
Dubnau, D. A. and Pollock, M. R. (1965) J. Gen. Microbiol. **41**, 7–21
Frère, J. M. and Joris, B. (1985) CRC Crit. Rev. Microbiol. **11**, 299–396
Ghuysen, J. M. (1991) Annu. Rev. Microbiol. **45**, 37–65
Masson, J. M. and Labia, R. (1983) Anal. Biochem. **128**, 164–168
Schwarz, U., Seeger, K, Wengenmayer, F. and Strecker, H. (1981) FEMS Microbiol. Lett. **10**, 107–109
Spratt, B. G. (1977) Eur. J. Biochem. **72**, 341–352
Spratt, B. G. and Croomie, K. D. (1988) Rev. Infect. Dis. **10**, 699–711
Spratt, B. G. and Pardee, A. B. (1975) Nature (London) **254**, 516–517

Received 31 December 1992/21 January 1993; accepted 25 January 1993