Kinetic properties of the *Bacillus licheniformis* penicillin-binding proteins

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In the analysis of the interactions between β -lactam antibiotics and their target enzymes, it is often difficult to estimate the kinetic properties of the molecules which react rapidly with their targets and in consequence behave as the most efficient antibiotics. The combined utilization of fluorescein-labelled penicillins and of a new competition method has allowed an accurate determination of the high second-order rate constants characterizing the acylation of *Bacillus licheniformis* penicillin-

binding protein 1 (PBP1) by penicillins and cephalosporins. Strategies were devised for measuring high acylation rates while avoiding titration effects. The method was also suitable for measuring the PBP kinetic parameters in intact cells. These results also confirmed that PBP1 is probably the main target of most β -lactam antibiotics. Cephalixin, however, reacted faster with PBP3.

INTRODUCTION

Penicillin-binding proteins (PBPs), usually bound to the cytoplasmic membranes of bacteria, participate in the assembly and constant remodelling of the wall peptidoglycan [1]. They are the targets of β -lactam antibiotics with which they interact according to the three-step pathway involving the formation of a rather stable covalent acyl-enzyme intermediate (EC^*), depicted in Scheme 1.

Scheme 1 Interaction of PBPs with β -lactam antibiotics

E is the PBP, C is the antibiotic, $E \cdot C$ is a non-covalent Henri-Michaelis complex whose dissociation constant is K , and P is the degraded and inactive antibiotic.

The affinity of a PBP for a β -lactam compound or the inactivating potency of the antibiotic towards a PBP is most often measured as the concentration of antibiotic (ID_{50}) required to achieve 50% saturation of the PBP after a given time at a specified temperature. But this value, related to the kinetic parameters by a complex equation, renders the comparison of data obtained under different conditions difficult and, particularly when it is quite small, can reflect a titration effect rather than the real sensitivity of the PBP.

A rigorous approach [2] rests on the estimation of the second- (k_2/K) and first-order (k_3) rate constants characterizing respectively the formation and breakdown of the acyl-enzyme intermediate (large k_2/K and small k_3 values result in efficient inactivation), and in consequence present a situation for the evaluation of the physiological importance of the various PBPs.

The rates of acylation and deacylation are usually determined either directly with radioactive antibiotics or by counter-labelling in the case of unlabelled compounds. In practice, these methods are unreliable when the acylation reaction is very fast, i.e. when

the PBP is very sensitive to the antibiotic. A new rapid, quantitative and highly sensitive method has been described recently in which PBPs were detected with the help of an ALF automatic DNA sequencer after reaction with fluorescein-labelled penicillins [3]. In addition, a very simple and efficient competition method has also been proposed, which should allow the easy determination of high k_2/K values [4]. In this paper, we have combined these two techniques to determine the kinetic parameters for the interaction between the *Bacillus licheniformis* PBPs and various penicillins, where the k_2/K values were sometimes larger than $10^2 \text{ mM}^{-1} \cdot \text{s}^{-1}$, and we show that they can be successfully utilized not only with isolated membranes but also the whole cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. licheniformis 749/110/C3 pen 22 strain (749/P22) was kindly given by Dr. J. Oliver Lampen (Waksman Institute, The State University of New Jersey, Piscataway, NJ, U.S.A.). It was obtained by submitting the wild-type strain of *B. licheniformis* 749/110 (749/I) to chemical mutagenesis [5,6] and produces a β -lactamase devoid of enzymic activity [7]. Cells were grown at 37 °C under orbital shaking (250 rev./min) in Luria-Bertani (LB) medium.

β -Lactam compounds

Cloxacillin was from Beecham Research Laboratories (Brentford, Middx., U.K.); cephaloglycin, cephalixin and cephalosporin C were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.); β -iodopenicillanic acid (β -IP) was from Pfizer Central Research (Sandwich, Kent, U.K.); benzylpenicillin was from Rhone-Poulenc (Paris, France); ceftazidime was from Glaxo Group Research (Greenford, Middx., U.K.); and cefotaxime was from Hoechst-Roussel (Romainville, France). All these compounds (for the structures see Matagne et al. [8]) were kindly given by the respective companies.

Abbreviations used: PBP, penicillin-binding protein; β -IP, β -iodopenicillanic acid; 7ACA, 7-aminocephalosporanic acid; Flu-6-APA, fluoresceyl-6-aminopenicillanic acid; Flu-Gly-6-APA, fluoresceyl-glycyl-6-APA; Flu-ampicillin, fluoresceyl-ampicillin; FLUOS, 5-[6]-carboxyfluorescein-*N*-hydroxy-succinimide ester.

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7-Aminocephalosporanic acid (7ACA) was purchased from Janssen Pharmaceutica (Beerse, Belgium). [^{14}C]Benzylpenicillin (54 mCi/mmol) and [^3H]benzylpenicillin (10 $\mu\text{Ci}/\text{nmol}$) were from Amersham International, and benzyl[^{35}S]penicillin (0.5 $\mu\text{Ci}/\text{nmol}$) was from NEN Products, DuPont de Nemours (Brussels, Belgium).

Fluoresceyl-6-aminopenicillanic acid (Flu-6-APA), fluoresceyl-glycyl-6-APA (Flu-Gly-6-APA) and fluoresceyl-ampicillin (Flu-ampicillin) were prepared as described by Lakaye et al. [9] and were used as mixtures of the 5' and 6' isomers.

Membranes

Membranes were prepared as described in [10], then washed twice in 40 mM sodium phosphate buffer, pH 7, containing 1 mM MgCl_2 and 5% (v/v) glycerol and suspended in the same buffer containing 0.1 mM PMSF (Boehringer, Mannheim, Germany). They were stored at -20°C (10 mg of total protein/ml). The protein concentration was estimated by the BCA method (Pierce, Rockford, IL, U.S.A.) [11] with BSA as standard.

Whole cells

Cultures were grown to an A_{550} value of 1.0, and after centrifugation the cells were resuspended in 40 mM sodium phosphate buffer, pH 7. The final volume of the cell suspension was about 5% of that of the original culture.

Estimation of cell density

Cultures were diluted in cold fresh medium to concentrations suitable for counting the colonies on LB agar plates after overnight incubation at 37°C . It was important to dilute the cultures in medium since utilization of water for that purpose resulted in a significant degree of cell lysis (about 50% after 30 min at 37°C). An A_{550} value of 1.0 corresponded to a cell density of 0.8×10^8 viable cells/ml of culture in LB medium. Note that this value strongly depended on the growth medium and on the wavelength utilized to measure the absorbance of the culture.

Determination of kinetic parameters

The k_2/K and k_3 parameters can be computed from the time courses of formation and breakdown of the acyl-enzyme [2]. A valid analysis rests on the following preliminary conditions. (1) No β -lactamase activity must be present. The very low activity found in the membranes of strain 749/P22 was inactivated by 5 μM β -IP [12], which does not significantly react with the *B. licheniformis* PBPs at this concentration. (2) For acylation studies, the β -lactam concentration should be at least five times greater than the sum of the concentrations of all the PBPs. (3) The PBPs under study should remain stable during the time needed for the experiment, i.e. a control sample incubated under the same conditions but in the absence of β -lactam should retain 100% of its penicillin-binding capacity. (4) The k_3 value should first be estimated, since the apparent pseudo-first-order rate constant for EC* formation (k_t) is given by eqn. (1) [2]:

$$k_t = k_3 + k_i \quad (1)$$

where

$$k_t = k_2[\text{C}]/(\text{K} + [\text{C}]) \quad (2)$$

Determination of k_3 values

The breakdown of the acyl-enzyme intermediate obeys eqn. (3) [13]:

$$\ln([\text{EC}^*]_t/[\text{EC}^*]_0) = -k_3 \cdot t \quad (3)$$

where $[\text{EC}^*]_0$ is the initial concentration of acyl-enzyme and $[\text{EC}^*]_t$ is its concentration at time t .

Labelled antibiotics

Membranes were quickly saturated with an excess of labelled antibiotic and the suspensions were incubated with a sufficient quantity of a suitable β -lactamase for hydrolysing the free antibiotic within 2–3 min. Samples were subsequently withdrawn after various periods of time and the reaction terminated by addition of 30% (v/v) of a denaturing mixture (1 M Tris/HCl buffer, pH 6.8, containing 4% (w/v) SDS, 31% (v/v) glycerol, 0.02% (w/v) Bromophenol Blue, 18% (v/v) mercaptoethanol and 0.01 M benzylpenicillin). The samples were heated at 100°C for 2 min and the PBPs were separated by migration on SDS/PAGE [14], with acrylamide/bisacrylamide ratios of 37.5:1 and 60:1 for the stacking and separating gels respectively. Depending on the M_r of the proteins, 8, 9 or 10% acrylamide separating gels were used on a 10 cm $\times$ 10 cm Mini-Protean II (Bio-Rad) or a 16 cm $\times$ 18 cm or 24 cm $\times$ 18 cm LKB 2001 (Pharmacia, Uppsala, Sweden) electrophoresis cell.

The Coomassie Blue-stained gels were dried at 60°C , submitted to fluorography [15] and exposed for 1–15 days at -70°C . Detection and quantification of the PBPs on the developed plates were carried out using a two-dimensional densitometer (Cybertech CS-1; Dalton, Waalwijk, The Netherlands).

Fluorescent samples were analysed on the same types of gels (18 cm $\times$ 30 cm) using an ALF DNA sequencer [16], where the quantification of the labelled protein is performed during the electrophoretic run (for details, see Galleni et al. [3]). In addition to the usual experimental errors, each ALF sequencer detector exhibits a somewhat different sensitivity. It was thus necessary to add an internal fluorescent standard to each sample. For this purpose BSA, whose M_r is intermediate between those of PBPs 3 and 4, was coupled to 5-[6]-carboxyfluorescein-*N*-hydroxy-succinimide ester (FLUOS) (Boehringer Mannheim Biochemica) in a 1:1 protein:FLUOS molar ratio as described by the supplier. The *Actinomadura* R39 DD-peptidase, saturated by Flu-Gly-6-APA according to [9], was alternatively added to the denaturing mixture. Quantification was carried out using Fragment Manager software (Pharmacia, Uppsala, Sweden).

Unlabelled antibiotics

The membranes were rapidly saturated and the excess of free antibiotic was usually eliminated by addition of *Enterobacter cloacae* 908R class C β -lactamase [17,18]. After increasing periods of time, samples were withdrawn and treated with Flu-ampicillin at a final concentration of 3 μM , which rapidly saturated the free PBPs. Note that this compound is not sensitive to the class C β -lactamase (D. Monnaie, B. Lakaye and J.-M. Frère, unpublished work). Alternatively, with cloxacillin, the class A β -lactamase of *B. licheniformis* was used and subsequently inactivated by β -IP [12].

Determination of k_2/K values

Three methods were used at 37°C , in 40 mM sodium phosphate buffer, pH 7: (A) Direct binding of an isotope- or fluorescein-

Scheme 2 Competition between labelled (L) and unlabelled (U) antibiotics for binding to a PBP

labelled antibiotic; (B) counter-labelling for unlabelled compounds; and (C) competition between unlabelled and labelled antibiotics.

Direct binding of a labelled antibiotic (Method A)

Accumulation of the acyl-enzyme intermediate proceeds according to eqn. (4) [19]:

$$[EC^*]/[E]_0 = [k_t/(k_t + k_3)] [1 - e^{-(k_t + k_3)t}] \quad (4)$$

but the data are more easily analysed on the basis of eqn. (5):

$$(\epsilon - \epsilon_{ss})/(\epsilon_0 - \epsilon_{ss}) = e^{-(k_t + k_3)t} \quad (5)$$

where $\epsilon = [E]_0 - [EC^*]$ and ϵ_0 and ϵ_{ss} are the ϵ values at time 0 and when the steady state has been reached respectively. In most cases, ϵ_{ss} was negligible when compared with ϵ_0 .

After addition of BSA-FLUOS, membranes were incubated with the labelled antibiotic. Samples were withdrawn after increasing periods of time and added to the denaturing mixture. The reaction time course was followed until a steady state was

reached. In most cases, this corresponded to complete saturation of the PBPs by the fluorescent antibiotic (low k_3 values).

Unlabelled antibiotics (Method B)

The procedure was similar to that described above, but after incubation with the unlabelled antibiotic U, the residual free enzyme was rapidly saturated with an excess of labelled antibiotic L, which directly yielded the ϵ value. The conditions were such that $[k_t]_L \gg [k_t]_U$.

Competition between two antibiotics (Method C)

Incubation of a PBP with a mixture of labelled (L) and unlabelled (U) antibiotics results in competition according to Scheme 2. If the k_3 steps can be neglected, i.e. if the incubation time is much shorter than the half-lives of both acyl-enzymes, it can be shown [4] that eqn. (6) holds until the free PBP has completely disappeared:

$$[EU^*]/[EL^*] = [k_t]_U/[k_t]_L = ([k_2/K]_U \cdot [U])/([k_2/K]_L \cdot [L]) \quad (6)$$

Eqn. (6) shows that the $[EU^*]/[EL^*]$ ratio is directly proportional to $[U]/[L]$. Thus, by increasing $[U]$ and $[L]$ by the same factor, the rate of the reaction can be increased without modification of the $[EU^*]/[EL^*]$ ratio, and complete saturation of the PBP can occur within a very short time. The $[EU^*]$ value can be obtained as the difference between the $[EL^*]$ values obtained in the absence ($[EL^*]_0$) and in the presence of U, i.e. $[EU^*] = [EL^*]_0 - [EL^*]$. In consequence, if $[k_t]_L$ is known, $[k_t]_U$ can be easily determined.

This method is by far the easiest, because eqn. (6) holds at all antibiotic concentrations provided that $[L]$ and $[U]$ are $\gg [E]_0$. Moreover, since the $[EU^*]/[EL^*]$ ratio is not time-dependent, it

Table 1 Acylation rate constants (k_2/K ; mM⁻¹·s⁻¹) and deacylation rate constants (k_3 ; s⁻¹) of the four PBPs of *B. licheniformis* 749/P22 with various β -lactams

A, Determined by direct binding of a labelled antibiotic (Method A); B, determined by counter-labelling with Flu-ampicillin (Method B); C, determined by competition (Method C). The k_3 values for the labelled (fluorescent or radioactive) compounds were measured directly, and the others were measured by counter-labelling with Flu-ampicillin. N.D., not determined.

Antibiotics	PBP1	PBP2	PBP3	PBP4
Penicillins				
Benzylpenicillin				
k_2/K	331 ± 37 (C)	0.225 ± 0.032 (A)	1.1 ± 0.5 (A)	2.3 ± 1.0 (A)
k_3	(2.1 ± 0.6) × 10 ⁻³	< 2 × 10 ⁻⁵	< 2 × 10 ⁻⁵	< 2 × 10 ⁻⁵
Flu-ampicillin				
k_2/K	367 ± 18 (A)	0.641 ± 0.081 (A)	12 ± 5 (A)	0.67 ± 0.3 (A)
k_3	(3.3 ± 0.5) × 10 ⁻⁴	< 2.6 × 10 ⁻⁴	< 2.6 × 10 ⁻⁴	N.D.
Flu-Gly-6-APA				
k_2/K	181 ± 76 (A and C)	0.016 ± 0.002 (A)	8 ± 1 (A)	3.0 ± 0.6 (A)
k_3	(6.5 ± 2.0) × 10 ⁻⁴	< 6.4 × 10 ⁻⁵	< 6.4 × 10 ⁻⁵	< 6.4 × 10 ⁻⁵
Cloxacillin				
k_2/K	165 ± 33	N.D.	N.D.	N.D.
k_3	< 10 ⁻⁴	N.D.	N.D.	N.D.
Cephalosporins				
Cephalexin				
k_2/K	30 ± 3 (C)	< 10 (C)	< 3 (C)	< 3 (C)
k_3	< 10 ⁻⁴	N.D.	N.D.	N.D.
Cephalexin C				
k_2/K	0.78 ± 0.04 (C)	2.6 ± 1.2 (C)	23 (C)	< 0.1 (C)
k_3	< 10 ⁻⁴	< 10 ⁻⁴	< 10 ⁻⁴	N.D.
Cefazidime				
k_2/K	0.85 ± 0.03 (B)	N.D.	N.D.	N.D.
k_3	< 5 × 10 ⁻⁵	N.D.	N.D.	N.D.
Ceftazidime				
k_2/K	285 ± 27 (B and C)	< 50	< 2	< 0.05
k_3	< 10 ⁻⁴	N.D.	N.D.	N.D.

Table 2 Comparison of the k_2/K values for the acylation of *B. licheniformis* PBP1 by some β -lactams as determined with whole cells and with isolated membranes

A, Determined by direct binding of a labelled antibiotic (Method A); B, determined by counter-labelling with Flu-ampicillin (Method B); C, determined by competition (Method C).

Antibiotics	k_2/K ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	
	Whole cells	Membranes
Penicillins		
Benzylpenicillin	370 ± 90 (C)	330 ± 40 (C)
Flu-Gly-6-APA	259 ± 11 (A)	180 ± 80 (C)
Cloxacillin	160 ± 30 (B)	165 ± 30 (C)
Cephalosporins		
Cephaloglycin	40 ± 4 (B)	30 ± 3 (C)
Ceftazidime	220 ± 40 (B)	280 ± 30 (B and C)
Cefotaxime	650 ± 150 (C)	600 ± 100 (C)

eliminates the need for withdrawing samples after increasing periods of time and allows the measurement of high k_2/K values by choosing a suitable $[U]/[L]$ ratio. In practice, the samples were usually incubated with the antibiotic mixture for a maximum of 3 min and the reaction was terminated by addition of the denaturing mixture.

Estimation of the number of PBPs per cell

Cells corresponding to 50 ml of a culture with an A_{550} of 1.0 were centrifuged and resuspended as above in 200 μl of buffer; 50 μM [^{35}S]benzylpenicillin was added and the samples were incubated for 20 min at 37 °C. Controls were first saturated by reaction with 50 μM unlabelled benzylpenicillin for 20 min at 37 °C. The samples were acidified by addition of 100 μl of 6 M HCl and extracted with 600 μl of butanol. The aqueous phase was treated with 200 μl of sodium phosphate buffer, pH 7, and 200 μl of a mixture of 50% (w/v) trichloroacetic acid and 3.7% (w/v) tungstosilicic acid in water, and allowed to stand for 10 min at 0 °C [20]. After centrifugation, the pellet was solubilized in 1 ml of 1% SDS and mixed with 10 ml of Ecoscint H (National Diagnostics, Manville, NJ, U.S.A.), and radioactivity was counted. Alternatively, the cell suspension was treated with 10 μM Flu-Gly-6APA for 5 min at 37 °C and analysed with the ALF DNA sequencer as described above. The Flu-Gly-6-APA labelled *Actinomadura* R39 DD-peptidase was added as an internal standard [3]. A total number of 29000 ± 5000 molecules per cell was found. In all cases, the experimental errors are given as S.D. values.

RESULTS AND DISCUSSION

According to Reynolds and co-workers, the *B. licheniformis* PBP1 behaves as a peptidoglycan transpeptidase. It is much more sensitive to benzylpenicillin than the three other PBPs of this species, and was the sole PBP to be significantly acylated at benzylpenicillin concentrations sufficient to induce cellular lysis [10]. In their studies, the acylation rates of PBPs 2, 3 and 4 could be approximated, but this was not possible for PBP1 because of a very high k_2/K value, resulting in a titration effect and, in consequence, to an unexpectedly high k_3 value.

In the present work, two strategies were utilized to determine high k_2/K values. (i) The high sensitivity of the ALF fluorescence detectors allowed the direct monitoring of the acylation time courses with the fluorescent antibiotics (method A). Indeed, the

Figure 1 Comparison of the classical fluorography method with the detection of fluorescent PBPs using the ALF sequencer

(a) SDS/PAGE and fluorography of benzyl[^{35}S]penicillin-labelled membranes of *B. licheniformis* 749/P22 strain (50 μg of total protein). The acrylamide concentration was 8% (w/v). The exposure time was 1 week. The negative was scanned which yielded the following proportions: PBP1, $18 \pm 3\%$ (i.e. about 5200 molecules); PBP2, $12 \pm 5\%$ (i.e. about 3500 molecules); PBP3, $2 \pm 1\%$ (i.e. about 600 molecules); PBP4, $68 \pm 6\%$ (i.e. about 20 000 molecules). Note that the proportion of PBP3 was sometimes slightly higher (up to 3%), suggesting a specific degradation of this protein in some experiments. (b) SDS/PAGE (10%) of whole cells of *B. licheniformis* 749/P22, treated with 10 μM Flu-Gly-6-APA for 5 min at 37 °C, on the ALF DNA sequencer. The adduct obtained by saturating the *Actinomadura* R39 DD-peptidase (M_r 50 000) by 2 μM Flu-Gly-6-APA was used as internal standard. The trace represents the direct recording of the PBP fluorescence as detected by the photomultipliers of the sequencer. The total number of PBP molecules per cell and their relative proportions determined by this technique are the same as those determined as described in (a). It should be noted, however, that in the experiment reported here saturation of the detector occurred with PBP4. The PBP4/PBP1 ratio was determined in a parallel run with a 4-fold dilution of the sample.

total amount of PBPs in the assays could be decreased, so that very low (10 nM) concentrations of antibiotics could be used which nevertheless remained significantly higher than the total PBP concentration. (ii) Alternatively, the k_2/K value for cephalosporin C was extracted from acylation time courses monitored using method B. This was made possible by the fact

that cephalosporin C was a rather poor acylating agent which reacted relatively slowly even at $[C]_0 \gg [E]_0$.

The k_2/K value for one fluorescent compound was then verified in a competition experiment with cephalosporin C (method C). Values of $209 \pm 54 \text{ mM}^{-1} \cdot \text{s}^{-1}$ ($n = 5$) or $209\,000 \pm 54\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $143 \pm 27 \text{ mM}^{-1} \cdot \text{s}^{-1}$ ($n = 4$) were found by directly monitoring the acylation time course with Flu-Gly-6-APA and by competition with cephalosporin C respectively. The values given in Table 1 are the means of the nine individual measurements. The competition method was subsequently used to determine the acylation parameters of all unlabelled compounds, with Flu-Gly-6-APA as the reference β -lactam.

Similarly, for ceftazidime the k_2/K value was obtained by counter-labelling with Flu-ampicillin using a very low quantity of membranes ($k_2/K = 290 \text{ mM}^{-1} \cdot \text{s}^{-1}$) and by competition with Flu-ampicillin ($k_2/K = 280 \pm 27 \text{ mM}^{-1} \cdot \text{s}^{-1}$, $n = 2$). Both methods were thus in excellent agreement, but the competition method was much easier to use. Note that for benzylpenicillin the low values for PBPs 2, 3 and 4 could be measured by directly monitoring the acylation time courses with labelled molecules (^{35}S , ^{14}C or ^3H) but that the high k_2/K value for PBP1 could only be measured by competition with Flu-Gly-6-APA.

The results summarized in Table 1 show that PBP1 is usually the most sensitive PBP, exhibiting k_2/K values greater than $10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ with several compounds, making it the likely lethal target. The only exception is cephalixin, which reacts 30-fold faster with PBP3, a result in agreement with that of Rodríguez-Tébar et al. [21], who identified PBP3 of *B. megaterium* as the probable lethal target for this cephalosporin. The only rather high k_3 value is that observed in the benzylpenicillin–PBP1 interaction. The 320 s half-life of this EC* complex is likely to complicate the analysis of the acylation time courses. In the present study, utilization of the competition method circumvented this difficulty, since complete acylation by a mixture of benzylpenicillin and reference fluorescent antibiotic could be obtained within a few seconds, i.e. significantly less than 10% of this half-life. The sensitivity of the ALF sequencer also allowed the detection of the PBPs in whole cells, and a comparison of the acylation rates of PBP1 in whole cells and isolated membranes (Table 2) demonstrates that the four PBPs found in isolated membranes can easily be visualized in whole cells (Figure 1). Moreover, the rates of acylation are similar for both types of sample, indicating the absence of a diffusion barrier *in vivo* between the PBPs and the culture medium in the Gram-positive *B. licheniformis*, at least in resting cells, thus contrasting with the situation in Gram-negative bacteria [22,23] and in some Gram-positives such as *Mycobacteria* [24].

In conclusion, the methods proposed in this study avoid the utilization of radioactive compounds and significantly decrease the time necessary for quantitative estimations. The high sen-

sitivity also allows experiments to be performed with whole cells, and very high k_2/K values can be determined even for unlabelled antibiotics by the competition technique. Two fluorescent compounds, Flu-Gly-6-APA and Flu-ampicillin, exhibit a very high affinity for PBP1, similar to that of benzylpenicillin. Moreover, Flu-ampicillin is very poorly hydrolysed by class C β -lactamases, which makes it an ideal counter-labelling agent in the determination of k_3 values for the antibiotics such as cephalixin, cephalosporin C and cephaloglycin which are very sensitive to these enzymes. By contrast, Flu-6-APA was a very poor inactivator, exhibiting low ($< 2 \text{ mM}^{-1} \cdot \text{s}^{-1}$) k_2/K values with all PBPs. Finally, the number of PBP molecules per cell was similar when determined with fluorescent or ^{35}S -labelled penicillins.

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