Penicillin-binding protein 2x of *Streptococcus pneumoniae*: enzymic activities and interactions with β -lactams

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The high-molecular-mass penicillin-binding protein (PBP) 2x, one of the primary targets of β -lactam antibiotics in *Streptococcus pneumoniae*, has been produced as a soluble form and purified in large amounts. It has been shown to catalyse hydrolysis and transfer reactions with different ester and thiolester substrates and its catalytic behaviour was often similar to that of the soluble DD-peptidase from *Streptomyces* R61. This provided an easy method to monitor the activity of the PBP. For the first time, a

reliable kinetic study of the interaction between a lethal target and β -lactam antibiotics has been performed. Characteristic kinetic parameters were obtained with different β -lactam compounds. These results not only validated the mechanism established with non-essential extracellular enzymes, but will also constitute the basis for comparative studies of the low-affinity variants from penicillin-resistant strains.

INTRODUCTION

The penicillin-binding proteins (PBPs) are the membrane-bound enzymes directly responsible for the sensitivity of bacteria to β -lactam antibiotics. Genes encoding the PBPs of different species have been cloned and sequenced. The deduced amino-acid sequences and functional analyses revealed three classes of high-molecular-mass PBPs (HMM-PBPs) (Ghuysen, 1991).

Streptococcus pneumoniae contains six PBPs (Hakenbeck et al., 1986). The functions of HMM-PBPs 1a, 1b, 2x, 2a and 2b (92–78 kDa) are unknown, whereas the 43 kDa PBP3 acts *in vitro* as a DD-carboxypeptidase (Hakenbeck and Kohiyama, 1982).

Strep. pneumoniae is an important pathogenic agent in man and, despite antibiotic therapy, remains a non-negligible cause of mortality (for a review, see Boulnois, 1992). It is one of the most penicillin-susceptible bacteria with a minimal inhibitory concentration of less than 10 ng/ml for benzylpenicillin. Accordingly, the penicillin-sensitivity of its primary target enzymes, the PBPs, appears to be very high when compared with that of *Escherichia coli* PBPs, for instance.

PBP 2x is one of the primary targets, since it is the first PBP changed into a low-affinity variant in β -lactam-resistant laboratory mutants (Laible and Hakenbeck, 1991), as well as in penicillin-resistant clinical isolates (Laible et al., 1991). It therefore represents a most suitable protein for studying the PBP– β -lactam interaction in detail. PBP 2x is a 750-amino-acid class-B PBP (82.35 kDa) with a C-terminal penicillin-binding domain closely related to those of *E. coli* PBP 3, *Neisseria gonorrhoeae* PBP 2 and *Strep. pneumoniae* PBP 2b, and with an N-terminal extension related to those of *E. coli* PBP 2 and methicillin-resistant *Staphylococcus aureus* PBP 2' (Laible et al., 1989). On the basis of homology searches and amino-acid alignments the conserved boxes Ser-Xaa-Xaa-Lys (337–340), Ser-Ser-Asn (395–397) and Lys-Ser-Gly (547–549) have been identified. Like other HMM-PBPs (Asoh et al., 1986; Bartholomé-DeBelder et al., 1988; Spratt and Cromie, 1988; Bowler and Spratt, 1989; Piras et al., 1990; El Kharroubi et al., 1991), PBP 2x is anchored

into the cytoplasmic membrane by an unprocessed N-terminal region (Leu-30–Ile-48) exhibiting a highly hydrophobic character.

HMM-PBPs have been produced as soluble proteins either through proteolytic treatment of isolated membranes (Piras et al., 1990; El Kharroubi et al., 1991) or by modification of the gene to remove the membrane anchor (Adachi et al., 1987; Bartholomé-DeBelder et al., 1988; Matsuzawa et al., 1988; Ernie et al., 1992). Usually, purified preparations have only been analysed by SDS/PAGE, a technique which would not reveal the presence of inactivated PBP molecules. PBP 2x of *Strep. pneumoniae* can be obtained in a soluble form by deleting the coding region for residues 19–48 (the soluble form is called PBP 2x*) and the construction of a high-level expression system in *E. coli* has facilitated purification of PBP 2x* in large amounts (Laible et al., 1992). Preliminary studies indicated that, as with other solubilized HMM-PBPs (Adam et al., 1991), this PBP catalyses hydrolysis and transfer reactions with chromogenic depsipeptide substrates (Laible et al., 1992).

In this paper, we propose a new protocol giving a better yield for the purification of PBP 2x* and we present the results of a detailed kinetic study of the interactions between PBP 2x* and different donor and acceptor substrates and β -lactam antibiotics.

MATERIALS AND METHODS

Chemicals and enzymes

The β -lactamase from *Bacillus licheniformis* was produced and purified in Liège as described by Matagne et al. (1990). Benzylpenicillin was from Rhône-Poulenc (Paris, France), ampicillin was from Bristol Benelux (Brussels, Belgium), cefotaxime was from Hoechst-Roussel (Romainville, France) and cephalixin was from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). The depsipeptide substrates were prepared in our laboratory as described previously (Adam et al., 1990, 1991). The structure of these compounds can be found in the same references. The tripeptide Ac₂-L-Lys-D-Ala-D-Ala was from UCB Bioproducts (Braine l'Alleud, Belgium). The amino acids were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Abbreviations used: PBP, penicillin-binding protein; HMM-PBPs, high-molecular-mass penicillin-binding proteins; [T]/[H], transfer/hydrolysis ratio; PBP 2x*, soluble form of *Strep. pneumoniae* PBP 2x.

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Plasmid

The plasmid pCG31-25 encoding the PBP 2x* from *Strep. pneumoniae* R6 was constructed by introducing a site-directed deletion in the *pbpX* gene by PCR technology (Laible et al., 1992).

Detection of PBP 2x*

During the purification procedure, PBP was routinely visualized by SDS/PAGE followed by Coomassie Blue staining or by fluorography after labelling with [¹⁴C]benzylpenicillin.

The concentration of PBP 2x* was determined by following the hydrolysis of *N*-benzoyl-D-alanylmercaptoacetic thiolester (substrate S2d) at 250 nm.

Production and purification

Step 1: Preparation of the crude enzyme

The enzyme was produced by *E. coli* DH5α harbouring the plasmid pCG31-25 and the crude extract was prepared as previously described (Laible et al., 1992) except that the cells (26 g wet wt.) were resuspended in 10 mM Tris/HCl buffer at pH 8.0 and that after the centrifugation step the supernatant was dialysed twice for 2 h against 10 mM Tris/HCl, pH 8.0. The crude extract (110 ml) contained 98 mg of PBP 2x* (as determined by following the hydrolysis of substrate S2d) and 3.9 g of total proteins.

Step 2: Q Fast-Flow column

The purification was performed with the help of a Pharmacia f.p.l.c. apparatus (Pharmacia, Uppsala, Sweden). The crude extract was adsorbed on a Q-Sepharose Fast-Flow column (2.6 cm × 14 cm) previously equilibrated with 10 mM Tris/HCl buffer, pH 8.0. The flow rate was 4 ml/min. The PBP was eluted with a linear gradient of the same buffer containing 1 M NaCl. The active fractions were collected and pooled. The resulting mixture was dialysed twice for 2 h against 10 mM sodium acetate adjusted to pH 5.0 with HCl.

Step 3: Mono S column

Five samples of the enzyme solution were adsorbed independently on a Mono S HR 5/5 column previously equilibrated with sodium acetate buffer adjusted to pH 5.0 with HCl. The flow rate was 1 ml/min. The PBP was eluted with a linear gradient of the same buffer containing 1 M NaCl. Three active fractions were collected and dialysed overnight against 10 mM sodium phosphate buffer, pH 7.0. The enzyme was conserved at -20 °C after addition of 10% (v/v) glycerol.

Isoelectric focusing and calculation of pI

The isoelectric points of the different forms of PBP 2x* were determined on a Multiphor II system (Pharmacia, Uppsala, Sweden) using a ready-made gel-ampholine polyacrylamide gel plate (pH 3.5–9.5).

The pI calculation was done with the help of the 'Isoelectric' algorithm from the GCG Analysis Software Package Version 6 (Devereux et al., 1984).

Determination of the kinetic parameters

All the experiments were performed at 37 °C in 10 mM sodium phosphate buffer using fraction II. Spectrophotometric measure-

ments were performed with a Uvikon 860 spectrophotometer linked to a microcomputer via an RS232 interface. Spectrofluorometric measurements were performed with a Perkin-Elmer LS 50 spectrofluorimeter (excitation and emission wavelengths were 280 nm and 342 nm respectively) and with a Biologic SFM3 stopped-flow apparatus as described in Jamin et al. (1991).

Reactions with the β-lactam antibiotics

Although it has never been clearly demonstrated, it is well accepted that the HMM-PBPs interact with β-lactam antibiotics according to the three-step scheme which was found to be valid for the soluble DD-peptidases from *Streptomyces* R61 and *Actinomadura* R39 (Frère et al., 1975; Fuad et al., 1976):

Scheme 1

where E = active enzyme, I = β-lactam compound, EI = Henri-Michaelis complex, EI* = acylenzyme, P = biologically inactive product, K = dissociation constant of the Henri-Michaelis complex, k_2 = first-order rate constant for the acylation step and k_3 = first-order rate constant for the deacylation step.

The k_2/K parameter, accounting for the acylation step efficiency, was measured by either using the substrate S2d as a reporter substrate (De Meester et al., 1987), or by following the decrease of the intrinsic fluorescence of the protein (Nieto et al., 1973; Frère et al., 1975) or by competition between [¹⁴C]benzylpenicillin and another unlabelled antibiotic (Frère et al., 1992). In the latter case, the ratio of labelled (with [¹⁴C]-benzylpenicillin) to unlabelled (with the other, non-radioactive β-lactam) acylenzyme was determined at different molar ratios of both antibiotics, by trapping the acylenzymes as described by Martin and Waley (1988) and by measuring the radioactivity bound to the protein. In these experiments, the k_2/K value of benzylpenicillin was used as a reference.

The k_3 parameter, accounting for the deacylation step, was determined by measuring the recovery of the PBP 2x* thiolesterase activity after inactivation with the β-lactam compound (Frère et al., 1974b). The PBP was inactivated by incubation with excess of antibiotic, the excess of antibiotic was eliminated by addition of the *B. licheniformis* β-lactamase and the progressive recovery of the thiolesterase activity was monitored with the help of substrate S2d. The k_3 value was deduced from the slope of the $\ln[(V_0 - V_t)/V_0]$ versus time plot where V_t and V_0 are the rates of thiolester hydrolysis at time t and after complete reactivation respectively.

The PBP 2x* concentration in the three fractions eluted from the Mono S column was determined by titration of the enzyme with benzylpenicillin (Frère et al., 1974a).

Reactions with the substrates

Usually, the disappearance of the substrate was directly monitored at 250 nm. The k_{cat} and K_m parameters were determined by recording the initial rates at different concentrations of substrate and by analysing these results according to the Hanes linearization of the Henri-Michaelis equation. In some cases, the K_m was so high that only k_{cat}/K_m could be determined.

In non-steady-state conditions, the apparent first-order rate constant for the formation of the acylenzyme was determined by recording the time course of the decrease of the intrinsic protein fluorescence with the help of the stopped-flow apparatus and by analysing these data with a non-linear regression analysis program (Leatherbarrow, 1987).

Table 1 Specificity of PBP 2x* for the acceptor substrates

In all cases, the donor substrate was 1 mM S2a and the potential acceptor was at a concentration of 10 mM. No transfer product and no acceleration of donor utilization were detected with glycylglycine, L-serine, L-alanine and α -Ac-L-Lys-D-Ala-D-Ala. With D-valine, no acceleration was observed but the product prepared with the help of the *Streptomyces* R61 D-peptidase eluted together with S2a itself. The retention time is that of the transfer product (T) and the [T]/[H] ratio is that of the areas of the peaks separated by h.p.l.c. (detection at 235 nm). The acceleration factor represents the increase in initial rate $\{(V_0/[E_0]) (s^{-1})\}$ divided by the acceptor concentration (0.01 M). The relative acceleration factor represents the acceleration factor divided by the initial rate measured in the absence of acceptor substrate. It is likely that a saturation effect is already present at this concentration with D-phenylalanine and that the acceleration factor is thus underestimated. The errors represent S.D. values (three measurements). Abbreviation: ND, not determined.

Acceptor substrate	Retention time (min)	Acceleration factor ($M^{-1} \cdot s^{-1}$)	Relative acceleration factor (M^{-1})	[T]/[H]
D-Alanine	13.9	7 ± 2	33 ± 10	1.0–1.3
D-Lactate	19	14 ± 2	67 ± 10	1.5–2.0
D-Histidine	6.2	15 ± 5	71 ± 24	3.0–3.3
D-Glutamine	7.9	< 2	< 10	0.2
D-Serine	7.8	< 2	< 10	0.06–0.13
D-Valine	19.6	< 2	< 10	ND
D-Leucine	20.3	< 2	< 10	0.7
D-Phenylalanine	20.5	21 ± 3	100 ± 14	4.0–4.9

Transfer/hydrolysis ratio ([T]/[H])

The residual substrate and the products were separated by h.p.l.c. on a LiChrospher 100 RP-18 (5 μ m particle diam.) column (Merck, Darmstadt, Germany). Eluent A was 10 mM sodium acetate adjusted to pH 3.0 with HCl and eluent B was acetonitrile. The flow rate was 1 ml/min. With substrate S2a, elution was performed under isocratic conditions (90:10, v/v) for 16 min, followed by an 8 min linear acetonitrile gradient (10–100%). The retention times for the hydrolysis product and for substrate S2a were 12.4 min and 19.5 min respectively. The retention times for the transfer products obtained with various acceptor substrates are presented in Table 1.

With substrate S2d, elution was performed under isocratic conditions (90:20, v/v) for 16 min, followed by a 4 min linear acetonitrile gradient (20–100%). The retention times for the transfer product (with D-alanine as acceptor), for the hydrolysis product, and for substrate S2d were 5.7, 6.6 and 13.2 min, respectively.

RESULTS

Purification

Figure 1 represents the elution profile of the Mono S column. The yields were about 80% with the first column (Q-Sepharose Fast-Flow) and about 85% with the second column (Mono S). The global yield of the purification was about 68%.

Fractions I, II and III, collected after the Mono S column step, represented 26%, 44% and 30% of the purified PBP 2x* respectively.

Physicochemical properties of PBP 2x*

The maximum of the u.v. absorption spectrum was at 277.5 ± 0.1 nm. From the known molecular mass and tyrosine and tryptophan contents of the protein (Laible et al., 1989), the value of $A_{1\%}^{1\text{cm}}$ was calculated to be 8.6 (Cantor and Schimmel,

1980). Titration of fraction II by benzylpenicillin was performed. Figure 2 shows the decrease of PBP 2x* activity in the presence of increasing concentrations of benzylpenicillin. Assuming that penicillin binds to the enzyme in a 1:1 ratio, the concentration of the active enzyme is equal to the minimum concentration of antibiotic which totally inhibits the enzyme. Using this concentration a value for $A_{1\%}^{1\text{cm}}$ was deduced from the absorption spectrum to be 8.0, which is in good agreement with the computed value. A similar agreement between the absorbance and the titration results was found for the two other fractions.

Figure 1 Elution profile of the Mono S column

PBP 2x* (13.5 mg) in 10 ml were adsorbed on the column at pH 5.0. Elution was performed by the salt gradient represented by the broken line. The three hatched areas represent the three fractions which were collected. The total yield of this step was 85%.

Figure 2 Titration of fraction II with benzylpenicillin, based on the inhibition of enzyme activity

PBP 2x* (5 μ l) was incubated for 15 min at 37 °C in the presence of increasing amounts of benzylpenicillin in a total volume of 80 μ l. The residual activity was determined by following the hydrolysis of a 200 μ M S2d solution. The molar ratio was calculated on the basis of a molecular mass of 79 kDa for PBP 2x*. The absorbance at 280 nm of the solution of enzyme was 1.17.

Table 2 Kinetic parameters for the hydrolysis of the depsipeptide substrates by *Strep. pneumoniae* PBP 2x*

General structure of the substrates is $C_6H_5-CO-NH-CHR^1-CO-X-C^*HR^2-COO^-$. Sle: $R^1 = -H$, $R^2 = -CH_2-C_6H_5$, $X = O$; S2a: $R^1 = -H$, $R^2 = -H$, $X = S$; S2c: $R^1 = -H$, $R^2 = -CH_3$, $X = S$; S2d: $R^1 = -CH_3$, $R^2 = -H$, $X = S$; S2e: $R^1 = -CH_3$, $R^2 = -CH_3$, $X = S$. Compounds S1e and S2c were racemic mixtures (C^* being asymmetric). The enzyme only utilized the D-isomer. Abbreviation: ND, not determined. The S.E.M. values are those obtained after directly fitting all the experimental data (three initial rate measurements for each substrate concentration and at least five different concentrations for each substrate) to the Henri-Michaelis equation.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
S1e	0.33 ± 0.09	2.8 ± 1.0	120 ± 80 (†) 80 ± 5 (§)
S2a	0.47 ± 0.04	0.8 ± 0.1	610 ± 150 (†) 450 ± 20 (§)
S2c	0.40 ± 0.04	0.13 ± 0.03	3200 ± 1000 (†)
S2d	30 ± 5	5.6 ± 0.9	5000 ± 1400 (†) 4200 ± 100 (§)
S2e	ND	ND	4900 ± 200 (§)

(†) k_{cat}/K_m computed from the individual values of k_{cat} and K_m .

(§) k_{cat}/K_m determined in first-order conditions ($[S] \ll K_m$).

(§) k_{cat}/K_m determined in non-steady-state conditions by directly following the accumulation of acylenzyme with the help of the stopped-flow apparatus.

Figure 3 pH-dependence (a) and variation with ionic strength (b) of the k_{cat}/K_m parameter for the hydrolysis of substrate S2d

(a) The buffers were as follows: 10 mM sodium cacodylate/HCl, pH 6.0; 10 mM sodium phosphate, pH 7.0; 10 mM sodium phosphate, pH 8.0 and 10 mM potassium borate, pH 9.0. The ionic strength was adjusted by adding NaCl. (b) The ionic strength was varied either by using different concentrations of sodium phosphate buffer, pH 7.0 (0.01 to 0.25 M) (○) or by adding NaCl (0.02 to 1.00 M) (●) to 10 mM sodium phosphate buffer, pH 7.0. The effects of concentration of phosphate buffer were similar with those obtained with NaCl.

Figure 4 Variation of the initial rate of S2a hydrolysis with the concentration of D-alanine

The final concentrations were 1 mM substrate S2a and 0.35 μ M PBP 2x*.

The excitation and emission fluorescence spectra exhibited maxima at 280 nm and at 342 nm respectively. No modification of the position of these maxima was observed during the interactions with the antibiotics and with the substrates.

The isoelectric focusing of the different fractions obtained after the Mono S column chromatography revealed the presence of two components in each fraction with isoelectric points equal to 4.9 ± 0.1 and 4.8 ± 0.1 respectively. It was surprising to find proteins exhibiting similar isoelectric points in fractions separated on the basis of the electric charge. Moreover, the calculated value for the protein, as calculated on the basis of the complete sequence deduced from the gene structure, was 5.6. These problems are currently under investigation.

Reactions with the substrates

(a) Hydrolysis reactions

As observed with other HMM-PBPs, no hydrolysis of the synthetic tripeptide $Ac_2-L-Lys-D-Ala-D-Ala$ (1.9 mM) was detected after 4 h of incubation at 37 °C in the presence of 0.3 μ M PBP 2x*.

Table 2 shows the parameters determined with various ester and thiolester substrates. Usually fraction II (1.5 mg/ml) was used for the kinetic studies. Note that the specific activity of substrate S2d was not significantly different in fractions I and III, showing that the three fractions contained proteins of similar catalytic properties. Similar k_{cat}/K_m values were obtained with the previously purified small sample (Laible et al., 1992) which contained all the different isoforms of the enzyme.

Figures 3(a) and 3(b) show the influence of pH and of the ionic strength on the second-order rate constant k_{cat}/K_m for the hydrolysis of substrate S2d.

(b) Concomitant hydrolysis and transfer reactions

In the presence of an acceptor substrate concomitant hydrolysis and transfer reactions occurred, as shown by separating the products and the substrate by h.p.l.c.

When the S2a concentration (1 mM) was larger than the K_m value (0.8 ± 0.1 mM), an increase in the initial rate of substrate disappearance was observed in the presence of different acceptor substrates. With D-alanine, a saturation curve was obtained as shown in Figure 4. With the other acceptor substrates, an acceleration factor was roughly determined (Table 1). The

Figure 5 Variation of the $[T]/[H]$ ratio with the acceptor substrate (D-alanine) and with the donor substrate (S2d) concentrations

The final concentrations of the donor substrate were as follows: [S2a] = 200 μM (○), 500 μM (●), 800 μM (□), 1200 μM (■) and 1600 μM (△). S.D.s were between 5% and 10%.

Figure 6 (a) Time course of the fluorescence decrease of PBP 2x* after mixing with substrate S2a in the stopped-flow apparatus at 37 °C and (b) variation of the apparent-first-order rate constant of fluorescence decrease with the concentration of S2a

(a) The final concentrations were 1 mM substrate and 0.6 μM PBP 2x*. (b) The line was fitted (simple weighting) to the equation: $k_{\text{app}} = 0.4 \text{ (s}^{-1}\text{)} + (k_2/K) \cdot [\text{S2a}]$. A value of $450 \pm 20 \text{ M}^{-1} \cdot \text{s}^{-1}$ was deduced for k_2/K (see Table 1). S.D.s were around 5%. The results obtained with a proportional weighting were not significantly different.

efficiency of the acceptor substrates was also investigated by measuring the $[T]/[H]$ ratios.

With substrate S2d, the $[T]/[H]$ ratio increased with the acceptor substrate concentration (Figure 5). It also appeared that, as observed with the DD-peptidases of *Streptomyces* R61

Table 3 Kinetic parameters for the interactions between the β -lactam antibiotics and PBP 2x* from *Strep. pneumoniae*

In experiments measuring the time-dependent decrease in fluorescence of the enzyme the rate of acylenzyme formation remained strictly proportional to the antibiotic concentration up to the following concentrations: benzylpenicillin, 10 μM ; cefotaxime, 40 μM (experiments performed in the stopped-flow apparatus); ampicillin, 1 μM ; and cephalixin, 20 μM . These values thus respectively represent minimum values of K for each of the considered antibiotics. In measurements determined by competition with [^{14}C]benzylpenicillin, ampicillin, cefotaxime or cephalixin was incubated with [^{14}C]benzylpenicillin (60 mCi/mmol; final concentration, 660 μM) at different molar ratios. The reactions were started by adding 20 μg of PBP 2X (total volume 50 μl). The errors represent S.D. values (at least six, three and six measurements for k_2/K , k_3 and % quenching respectively).

Antibiotics	k_2/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^5 \times k_3$ (s^{-1})	Quenching (%)
Penicillins			
Benzylpenicillin	$58\,000 \pm 5\,000$ (†)	5.5 ± 0.5	8.9 ± 0.3
Ampicillin	$67\,000 \pm 2\,000$ (†) $70\,000 \pm 12\,000$ (§)	4.6 ± 1.0	11.4 ± 1.5
Cephalosporins			
Cefotaxime	$162\,000 \pm 4\,000$ (†) $130\,000 \pm 21\,000$ (§)	0.10 ± 0.05	20 ± 4
Cephalixin	1600 ± 100 (*) 1400 ± 400 (†) 1700 ± 600 (§)	2.1 ± 0.4	7.5 ± 1.0

(*) Determined by using S2d as reporter substrate.

(†) Determined by following the time-dependent fluorescence decrease of the enzyme.

(§) Determined by competition with [^{14}C]benzylpenicillin.

and *Actinomadura* R39 (Jamin M., Wilkin, J. M. and Frère, J. M., unpublished work), the $[T]/[H]$ ratio decreased when the S2d concentration increased.

Furthermore, a quenching of the intrinsic fluorescence of the PBP 2x* was observed during the interaction with substrate S2a (Figure 6a). The pseudo-first-order rate constant determined from the time course of fluorescence quenching varied linearly with the concentration of substrate S2a, yielding a second-order rate constant of $450 \pm 20 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figure 6b). This was in excellent agreement with the k_{cat}/K_m value determined for the hydrolysis of the same compound (Table 2). The results of Figure 6(b) also indicate values for $k_2 > 6 \text{ s}^{-1}$ and $K > 10 \text{ mM}$, results which were in agreement with a rate-limiting deacylation (at substrate saturation) as observed with the DD-peptidases of *Streptomyces* R61 and *Actinomadura* 39 (Jamin et al., 1991).

Reactions with the β -lactams

The binding of the β -lactam antibiotics causes quenching of the intrinsic fluorescence of the enzyme. The amplitude of the quenching depends on the tested compound (Table 3).

The values of k_2/K and k_3 obtained with different penicillins and cephalosporins are presented in Table 3. With benzylpenicillin and cefotaxime, the quenching of the fluorescence of the enzyme was monitored with the help of the stopped-flow apparatus. Figure 7(a) shows the time-course decrease of protein fluorescence after mixing with the cefotaxime. The k_2/K was deduced from the plot of the apparent rate constant for the quenching versus the cefotaxime concentration (Figure 7b).

The inactivation of PBP 2x* by cephalixin was also followed using thiolester S2d as reporter substrate. The $k_2/K = 1600 \pm 100 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained by this method, was in good agreement with the k_2/K value determined by following the quenching

Figure 7 (a) Time course of the decrease of fluorescence of PBP 2x* after mixing with cefotaxime in the stopped-flow apparatus at 37 °C and (b) variation of the apparent-first-order rate constant of decrease of fluorescence with the concentration of cefotaxime

(a) The final concentrations were 5 μM cefotaxime and 0.6 μM PBP 2x*. (b) The line was fitted to the equation $k_{\text{app}} = (k_2/K) \cdot [\text{cefotaxime}]$. A value of $162\,000 \pm 4000 \text{ M}^{-1} \cdot \text{s}^{-1}$ was deduced for k_2/K (see Table 3). The S.D.s were around 5%.

of the intrinsic fluorescence of the PBP ($1400 \pm 400 \text{ M}^{-1} \cdot \text{s}^{-1}$). These results were confirmed by a competition experiment between cephalexin and [^{14}C]benzylpenicillin ($k_2/K = 1700 \pm 600 \text{ M}^{-1} \cdot \text{s}^{-1}$).

DISCUSSION

Although numerous studies have been devoted to the interactions between PBPs and β -lactams, those for which accurate kinetic parameters are available remain a very select minority.

Scheme 1, involving the acylation of the active serine residue (Frère et al., 1976; Broome-Smith et al., 1985; Keck et al., 1985) and first proposed for model enzymes such as the *Streptomyces* R61 DD-peptidase (Frère et al., 1975), is now generally recognized as valid for all essential PBPs (Frère and Joris, 1985).

However, apart from a few low-molecular-mass PBPs and the soluble DD-peptidases from *Streptomyces* R61 and *Actinomadura* R39, most of the published values have been deduced from very unreliable measurements (Frère and Joris, 1985). This is particularly true for the HMM-PBPs, for which the absence of measurable enzymic activity has transformed the determination of the kinetic parameters involved in the acylation and deacylation phenomena into a kind of obstacle race.

In the present paper, we have obtained accurate values of these kinetic parameters for PBP 2x*. This was greatly facilitated by

two interesting observations: (1) the discovery of ester and thiolester substrates which can be utilized to easily visualize the enzymic activity (Adam et al., 1990, 1991; Laible et al., 1992); and (2) the possibility of directly monitoring the formation of the acylenzyme by following the time course of fluorescence decrease after addition of the antibiotic.

To obtain the large quantities of PBP 2x* needed to perform those studies, it was necessary to modify the purification method of Laible et al. (1992). Indeed, the replacement of the dye chromatography stage by an ion-exchange step allowed processing of 2 g of protein in one run instead of 28.5 mg. The final yield of the purification was 68%, a value which can be considered as very satisfactory. Moreover, the Mono S chromatography step indicated the presence in the preparation of three isoforms of the protein which could not be separated by SDS/PAGE; this is in agreement with the results described in the previous report (Laible et al., 1992) where the purified preparation appeared as a single band after SDS/PAGE. Accordingly, the titration experiments clearly showed that each fraction contained essentially pure PBP 2x*. Despite the presence of two components which could be separated on the basis of their isoelectric point values, all the kinetic experiments demonstrated that the various isoforms had identical enzymic properties. In consequence, we believe that here, for the first time, the demonstration of the purity of an HMM-PBP by a titration experiment has been performed.

The activity of PBP 2x* against some of the substrates was rather high, particularly when compared with the very low rate of polymerization of the natural precursor observed with *E. coli* HMM-PBPs (Nakagawa et al., 1984). Among the various HMM-PBPs which have been studied so far with the thiolester substrates (Adam et al., 1991), the highest $k_{\text{cat.}}/K_m$ value was obtained in the present study using substrate S2d (approx. $8000 \text{ M}^{-1} \cdot \text{s}^{-1}$). Moreover, the enzyme catalyses transpeptidation reactions with various acceptors and the disappearance of the thiolester can be followed at 250 nm.

As with the *Streptomyces* R61 DD-peptidase, formation of the acylenzyme appeared to induce some quenching of the intrinsic fluorescence of the protein (Nieto et al., 1973; Jamin et al., 1991). This was observed with both substrates and β -lactams. Accumulation of acylenzyme with the substrates indicated a rate-limiting deacylation which was confirmed by the similar $k_{\text{cat.}}$ values found for the three substrates which formed the same acylenzyme (S1e, S2a and S2c) and by the rate acceleration recorded upon addition of D-alanine and various D-amino-acid residues.

The catalysis of transfer reactions also offered some similarities with that observed with *Streptomyces* R61 DD-peptidase. Strikingly, the non-linearity of the $[T]/[H]$ ratio versus acceptor concentration and the dependence of this ratio on the donor concentration invalidate a simple model where the acylenzyme is partitioned between hydrolysis and transpeptidation pathways and where the tertiary acylenzyme-acceptor complex can only give rise to the transpeptidation product (Frère, 1973; Frère et al., 1973; Jamin et al., 1991). Similarly, D-histidine and D-phenylalanine were among the best acceptors for both enzymes. Conversely, D-glutamine, D-valine and D-leucine, which were good acceptors for R61 DD-peptidase, were barely utilized by PBP 2x*. A major difference between the two proteins was the absence of activity of PBP 2x* on the tripeptide $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$, which is the standard substrate for R61 DD-peptidase.

In conclusion, our results supply the first reliable kinetic parameters to have been determined for the interactions between a high-molecular-mass, essential PBP and β -lactam antibiotics. These results will represent the basis for a meaningful analysis of

the properties of PBP 2x* variants isolated from strains which have been selected for their resistance to β -lactams, and will in consequence constitute a cornerstone for a better understanding of 'intrinsic' resistance phenomena.

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