Biochemical characterization of the 49 kDa penicillin-binding protein of *Mycobacterium smegmatis*

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The 49 kDa penicillin-binding protein (PBP) of *Mycobacterium smegmatis* catalyses the hydrolysis of the peptide or *S*-ester bond of carbonyl donors $R_1\text{-CONH-CHR}_2\text{-COX-CHR}_3\text{-COO}^-$ (where X is NH or S). In the presence of a suitable amino acceptor, the reaction partitions between the transpeptidation and hydrolysis pathways, with the amino acceptor behaving as a simple alterna-

tive nucleophile at the level of the acyl-enzyme. By virtue of its N-terminal sequence similarity, the 49 kDa PBP represents one of the class of monofunctional low-molecular-mass PBPs. An immunologically related protein of M_r 52000 is present in *M. tuberculosis*. The 49 kDa PBP is sensitive towards amoxycillin, imipenem, flomoxef and cefoxitin.

INTRODUCTION

Bacteria possess three classes of penicillin-binding proteins (PBPs), which are essential membrane-bound cell-wall-synthesizing enzymes [1]. The high-molecular-mass PBPs of classes A and B are involved in wall peptidoglycan assembly and cell morphogenesis. These are multimodular proteins consisting of an N-terminal module fused to a C-terminal penicillin-binding module [2]. The low-molecular-mass PBPs are single catalytic entities performing DD-peptidase, esterase and thiol esterase activities [3]. The low-molecular-mass PBPs control the extent of wall peptidoglycan cross-linking and are involved in peptidoglycan remodelling throughout the life cycle of the cells [4].

Mycobacterial diseases, including tuberculosis, remain a cause of concern, particularly owing to the emergence of drug-resistant strains [5]. This has prompted a re-evaluation of the potential of existing antimicrobial agents including β -lactam antibiotics, in view of existing reports on the susceptibility of pathogenic mycobacteria *in vitro* and *in vivo* towards β -lactam antibiotics [6–9]. Efforts towards identifying the PBPs and their genes in mycobacterial species have been initiated in several laboratories [10–13]. We have purified a 49 kDa PBP from *Mycobacterium smegmatis* [10]. The kinetic properties of this PBP are presented here.

MATERIALS AND METHODS

Materials

Temocillin, cloxacillin, oxacillin and amoxycillin were gifts from Smith Kline Beecham; moxalactam, cefoxitin, cefotaxime, cephalixin, ceftazidime, piperacillin, methicillin, cefaclor and cefsulodin were from Sigma; flomoxef, cefmenoxime, ceftiofur and imipenem were gifts from Professor J. M. Frère (University of Liège, Liège, Belgium). The structures of the *S*-esters are given in Table 1. These were gifts from Professor Frère. The peptide $\text{Ac}_3\text{-L-Lys-D-Ala-D-Ala}$ and D-Ala were purchased from Sigma. UDP-*N*-acetylmuramyl-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala was a gift from Dr. Martine Nguyen-Disteche (University of Liège). [^3H]Benzylpenicillin and [^{14}C]benzyl-

penicillin were products of Amersham. All other chemicals were obtained from commercial sources.

N-terminal sequencing of the 49 kDa PBP and search for similarity with other PBPs

The 49 kDa PBP was purified as described by Basu et al. [10]. It was electroblotted on a poly(vinylidene difluoride) membrane and N-terminal sequencing was performed on an Applied Biosystems model 477A pulsed liquid sequenator in Ghent with on-line analysis of the phenylthiohydantoin-amino acids. Similarity searches were performed with the algorithm 'Pile Up' from the GCG package [14].

Raising of antibodies against the 49 kDa PBP, and Western blotting

Purified 49 kDa PBP (100 μg) was injected subcutaneously with incomplete Freund's adjuvant into one male rabbit. Successive injections with incomplete Freund's adjuvant (50 μg each) were repeated at the end of the second, third and fourth weeks, followed by bleeding at the end of the fifth week. Western blotting was performed as described by Towbin et al. [15].

Interactions of the 49 kDa PBP with β -lactam antibiotics, *S*-esters and peptides

This was interpreted on the basis of the following three-step scheme [16,17]:

where E is active enzyme, I is β -lactam compound, EI is Henri–Michaelis complex, EI^* is acyl-enzyme, P is biologically inactive product, K is the dissociation constant of the Henri–Michaelis complex, k_{+2}/K is the second-order rate constant for the acylation step and k_{+3} is the first-order rate constant for the deacylation step.

Table 1 Structures of the S-esters

Substrate	R ₂	R ₃
S2a	H	H
S2c	H	CH ₃
S2d	CH ₃	H

Reaction with the S-esters

The S-esters and the 49 kDa PBP were incubated in 10 mM sodium phosphate, pH 7, at 37 °C. The time-dependent decrease in absorbance was monitored at 250 nm with a UVIKON 860 spectrophotometer linked to a microcomputer via an RS232 interface. The k_{+2}/K values were computed by analysing the complete time course. The possible inhibition of the enzyme by the reaction products of hydrolysis was tested as described by Adam et al. [18]. The transpeptidation-to-hydrolysis ratios were determined by HPLC under initial rate conditions, i.e. before 10 % of the donor substrate had disappeared [18,19].

Reactions with the peptide substrates

The peptides and the 49 kDa PBP were incubated in 5 mM Tris/HCl, pH 7.2, at 37 °C. The released D-alanine was measured by the D-amino acid/peroxidase procedure [20].

Determination of the kinetic parameters k_{+2}/K and k_{+3} of interaction of the 49 kDa PBP with benzylpenicillin

This was done as described [21,22], with Ac₂-L-Lys-D-Ala-D-Ala as reporter substrate.

Determination of the kinetic properties of the 49 kDa PBP with non-radioactive β -lactams

Knowing the k_{+3} value of acyl-enzyme breakdown with the use of radioactive benzylpenicillin, we determined the k_{+2}/K value for non-radioactive β -lactams in a one-step competition with radioactive benzylpenicillin (for which the k_{+2}/K value was known) as described by Frère et al. [23] from the equation:

$$(k_{+2}/K)_1 = (k_{+2}/K)_2 \frac{[E-D^*_1][D_2]}{[E-D^*_2][D_1]}$$

The amount of radioactive acyl-enzyme formed with the β -lactam 2 in the absence ($[E-D^*_2]_0$) and in the presence ($[E-D^*_2]$) of the β -lactam 1, were measured by densitometric scanning of the fluorograms, and $[E-D^*_1] = [E-D^*_2]_0 - [E-D^*_2]$; $[D_1]$ and $[D_2] \gg [E_0]$. Incubations of the enzyme with antibiotics were performed for three different periods, with a maximum incubation time of 3 min so that the time of contact between the enzyme and the antibiotic was in each case short compared with the lifetime of the E-D* adducts. Under these conditions the ratios of $[E-D^*_1]/[E-D^*_2]$ were independent of the time of contact.

RESULTS

Determination of the N-terminal sequence and similarity search with other known PBPs

The N-terminal sequence of the 49 kDa PBP showed similarity with the family of bacterial monofunctional low-molecular-mass PBPs, the highest similarity being found with the PBP 5 of *Bacillus subtilis* (Figure 1). Western blotting with the anti-49 kDa antibody and membranes from *M. tuberculosis* gave a single cross-reactive band of M_r 52000.

Interaction with peptide and S-ester substrates

The 49 kDa PBP hydrolysed both the tripeptide Ac₂-L-Lys-D-Ala-D-Ala and the pentapeptide UDP-MurNAc-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala, the latter being a closer analogue of its natural substrate. The k_{cat}/K_m values were comparable (Table 2), suggesting that both substrates were hydrolysed with equal efficiency.

With the S-esters, the activity was highest with benzoyl-D-Ala-thioglycollate (S2d). The activity was diminished when R₂ and R₃ were both H and was negligible when R₂ was H and R₃ was CH₃ (Table 2). The R61 and R39 DD-peptidases preferentially hydrolyse D-Ala-D-Xaa C-termini [24,25]. The 49 kDa PBP of *M. smegmatis* seems to behave similarly. Benzoyl-D-Ala-thioglycollate was the preferred substrate for the DD-peptidases/PBPs from *Streptomyces* R61 and K15 and *Actinomodura* R39, and PBP2x from *Streptococcus pneumoniae* [26–29].

The hydrolysis of the 49 kDa PBP with S2d proceeded with a k_{cat} of 28.4 s⁻¹, a K_m of 0.11 mM and a k_{cat}/K_m of 250 000 M⁻¹·s⁻¹ in comparison with values of 430 000 and 336 000 M⁻¹·s⁻¹ for the R61 and R39 enzymes respectively [29]. No product inhibition was observed at concentrations corresponding to complete

Figure 1 Amino acid sequence alignments of the 49 kDa PBP (N-terminal sequence) with some low-molecular-mass PBPs of *Escherichia coli* and *Bacillus subtilis*

Table 2 Kinetic parameters for the hydrolysis of peptides and *S*-estersResults are means $\pm$ S.E.M. for three determinations.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Ac ₂ -L-Lys-D-Ala-D-Ala		7.4 ± 0.4	114 ± 11
UDP-MurNAc-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala		5.8 ± 0.3	152 ± 10
S2a			25000 ± 2000
S2c			54 ± 5
S2d	28 ± 1	0.11 ± 0.01	250000 ± 11000
S2d + D-Ala (50 mM)	134 ± 9	0.53 ± 0.03	$260000 \pm 3000^*$

* Similar results were obtained when derivations were made by analysis of complete time courses or by measuring initial rates and fitting the data to the Henri-Michaelis equation.

Figure 2 Effect of increasing concentrations of the amino acceptor D-alanine on the k_{cat} for the utilization of benzoyl-D-Ala-thioglycollate by the *M. smegmatis* 49 kDa PBP

hydrolysis of the substrates. Increasing concentrations of D-Ala caused a commensurate increase in both k_{cat} (Figure 2) and K_m , so that k_{cat}/K_m remained constant (results not shown) and equal to that of the hydrolysis pathway, indicating that the acceptor behaved as a simple alternate nucleophile at the level of the acyl-enzyme [17]. At a fixed concentration of benzoyl-D-Ala-thioglycollate (1 mM), the transpeptidation-to-hydrolysis ratio was directly proportional to the D-Ala concentration (results not shown).

Interaction of the 49 kDa PBP with β -lactam antibiotics

The value of k_{+2}/K obtained with radioactive benzylpenicillin was $130000 M^{-1} \cdot s^{-1}$, in comparison with values of 135, 180000 and $300000 M^{-1} \cdot s^{-1}$ for *Streptomyces* K15, R61 and *Actinomodura* R39 enzymes respectively. The value of k_{+3} was $10^{-4} s^{-1}$. The k_{+2}/K values indicate the acylating efficiency of the β -lactams. The affinity of the 49 kDa PBP was highest for amoxycillin (Table 3). The good inactivators include the oxacephems moxalactam and flomoxef, and the carbapenem imipenem. Although the 7-methoxycephalosporin cefoxitin was a good inhibitor, the 6-methoxypenicillin temocillin was not a good inactivator, both being β -lactamase-stable. Assuming that the 52 kDa PBP of *M. tuberculosis* that reacts with the anti-49 kDa antibody is the equivalent of this PBP, it is worth mentioning

Table 3 Efficacy of different β -lactam antibiotics against the 49 kDa PBPResults are means $\pm$ S.E.M. for three determinations.

Antibiotic	k_{+2}/K ($M^{-1} \cdot s^{-1}$)
Amoxycillin	49000 ± 5000
Imipenem	23000 ± 2000
Temocillin	4800 ± 400
Flomoxef	27000 ± 2000
Moxalactam	23000 ± 1000
Ceftizoxime	3000 ± 100
Cefmenoxime	7500 ± 400
Cephalexin	8700 ± 700
Ceftazidime	5000 ± 400
Ceftiofur	2500 ± 200
Cefaclor	3200 ± 200
Cefsulodin	2700 ± 100
Cefotaxime	1800 ± 50
Cefoxitin	20000 ± 2000
Cloxacillin	2500 ± 200
Oxacillin	1800 ± 100
Piperacillin	5700 ± 400
Methicillin	4300 ± 300

that the 52 kDa *M. tuberculosis* PBP was sensitive to imipenem and amoxycillin [11]. Previous reports had demonstrated that a 50 kDa PBP of *M. fortuitum* had the highest affinity for imipenem, followed by cefoxitin and amoxicillin [12]. The third-generation aminothiazolyl cephalosporins tested, and the isoxazolyl penicillins tested, were not efficient acylating agents. The acylureidopenicillin piperacillin was also not efficient.

DISCUSSION

To use β -lactam antibiotics at a clinical level, a detailed knowledge of their target sites, namely the PBPs, is essential. The characterization of the 49 kDa PBP of *M. smegmatis* is one of the first steps towards this end. Among the monofunctional low-molecular-mass PBPs, the R61 DD-peptidase and the K15 transpeptidase are the only two PBPs for which three-dimensional structural data and a detailed analysis of the transpeptidation reaction are available, both being soluble extracellular enzymes. The similarity of the 49 kDa PBP with PBP5 of *B. subtilis*, and the ability of this PBP to act on tri- and penta-peptide substrates, mark it as one of the low-molecular-mass PBPs of mycobacteria. Like other low-molecular-mass PBPs, this showed a preference for benzoyl-D-Ala-thioglycollate among the *S*-ester substrates tested. In the presence of suitable acceptor substrates, it catalyses concomitant carboxypeptidation and transpeptidation. Amoxycillin was found to be one of the most potent inactivators of this enzyme. The importance of this PBP is further highlighted by the presence of an immunologically related 52 kDa PBP in the slow-growing pathogenic *M. tuberculosis*. A 52 kDa PBP has been suggested to be one of the important β -lactam targets in *M. tuberculosis* [11].

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