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Acyltransferase activities of the high-molecular-mass essential penicillin-binding proteins

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The high-molecular-mass penicillin-binding proteins (HMM-PBPs), present in the cytoplasmic membranes of all eubacteria, are involved in important physiological events such as cell elongation, septation or shape determination. Up to now it has, however, been very difficult or impossible to study the catalytic properties of the HMM-PBPs *in vitro*. With simple substrates, we could demonstrate that several of these proteins could catalyse the hydrolysis of some thioesters or the transfer of their acyl moiety on the amino group of a suitable acceptor nucleophile. Many of the acyl-donor substrates were hippuric acid or benzoyl-D-alanine derivatives, and their spectroscopic properties enabled a direct monitoring of the enzymic reaction. In their presence, the binding of radioactive penicillin to the PBPs was also inhibited.

INTRODUCTION

The cytoplasmic membranes of bacteria contain multiple penicillin-binding proteins (PBPs) whose molecular-mass values range between 25 and 120 kDa. In most species, the low-molecular-mass (LMM) PBPs do not seem to play a major physiological role and behave *in vitro* mainly as D-alanyl-D-alanine carboxypeptidases (Frère & Joris, 1985). The only known exception is the 27 kDa PBP of *Streptomyces* K15, which exhibits a strict DD-transpeptidase activity with small synthetic peptides and esters and seems to be a lethal target for β-lactam antibiotics (Nguyen-Distèche *et al.*, 1986). The high-molecular-mass (HMM) PBPs (molecular mass > 50 kDa) appear to be of prime importance in physiological events such as cell elongation, septation or shape determination, and their inactivation by β-lactams leads to the appearance of aberrant morphologies and, ultimately, to cell death (Spratt, 1975, 1977; Spratt & Pardee, 1975). Different studies indicate that those HMM-PBPs are made of two distinct domains. Penicillin binds to the *C*-terminal domain, which is thus assumed to catalyse the transpeptidation reaction (Ghuysen, 1991). The *N*-terminal domain contains the membrane anchor and is responsible for the transglycosylase activity of *Escherichia coli* PBP1b during the synthesis of cross-linked peptidoglycan from the lipid-intermediate-linked disaccharide peptide (Suzuki *et al.*, 1980; Tamura *et al.*, 1980; Matsuhashi *et al.*, 1981). The situation with the other HMM-PBPs is less clear and, even with PBP1b, the experimental system is so complicated that it precludes any meaningful kinetic analysis of the transpeptidation reaction.

Thus, up to now, no simple system comparable to that utilized with the LMM DD-carboxypeptidases was available for assays *in vitro* of the HMM-PBPs.

We have recently described the synthesis of esters and thioesters which can be hydrolysed by various β-lactamases and LMM DD-peptidases (Adam *et al.*, 1990). When suitable α-amino or hydroxy acids of the D series were included in the assay mixtures, transpeptidation and transesterification reactions were observed (Jamin *et al.*, 1991). Moreover, the spectroscopic properties of these substrates were such that a direct spectrophotometric monitoring of the reactions could be performed (De Meester *et al.*, 1987; Adam *et al.*, 1990), which yielded an easy method for assaying possible transpeptidation activities of HMM-PBPs.

Here we show that various HMM-PBPs catalyse the transfer of the acyl moiety of some of these substrates on to suitable acceptor nucleophiles and that the same compounds successfully inhibit the binding of benzylpenicillin to these proteins.

MATERIAL AND METHODS

Substrates

The structures of the substrates are given in Fig. 1. The synthesis of compounds S1d, S2a, S2b, S2c and S3d was described previously (Adam *et al.*, 1990). Benzoyl-D-alanine was obtained by adding 0.95 equiv. of benzoyl chloride in dry tetrahydrofuran to 1 equiv. of D-alanine in water. The pH was maintained at 10 throughout the reaction. The product was precipitated by acidification to pH 3.0. Coupling with mercaptoacetic acid was performed as described (Adam *et al.*, 1990) for substrate S2a, and the product was purified by reverse-phase h.p.l.c. on a C_{18} column using a 0.1% trifluoroacetic acid (in water)/acetonitrile gradient. Substrate S2e was similarly obtained by using thiolactic acid instead of mercaptoacetic acid.

HMM-PBPs

We used soluble forms of HMM-PBPs obtained by genetic engineering or by proteolysis of the membranes. *Escherichia coli* PBP3p was a soluble derivative (60 kDa) of PBP3, where the 36 *N*-terminal residues of the precursor (63 kDa) have been replaced by the ompA signal peptide. The protein was secreted into the periplasm. SDS/PAGE of purified labelled PBP3p also yielded a band of lower molecular mass (37 kDa). This was probably due to the presence of a nick in a small proportion (15%) of the native protein, since the two native forms could not be separated on the basis of their sizes and exhibited the same sensitivity to penicillins (M. Adam, C. Piron-Fraipont & M. Nguyen-Distèche, unpublished work). *Streptococcus pneumoniae* PBP2Bc

Abbreviations used: HMM (LMM)-PBP(s), high-molecular-mass (low-molecular-mass) penicillin-binding protein(s).

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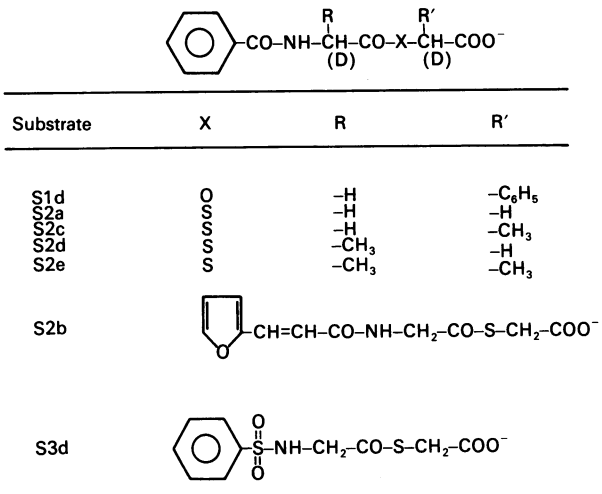

Fig. 1. Structures of the esters and thioesters

was a cytoplasmic soluble derivative (70 kDa) in which the 39 *N*-terminal residues of the precursor (77 kDa) have been replaced by the Met-Ser dipeptide (W. Keck & B. G. Spratt, unpublished work). *Enterococcus hirae* t-PBP1d and t-PBP3s (58 kDa) were soluble fragments respectively of PBP1 (120 kDa) and of PBP3s (77 kDa) obtained by trypsin digestion of Triton extracts of cytoplasmic membrane preparations (Piras *et al.*, 1990).

Kinetic data

Spectrophotometric measurements were performed with a Uvikon 860 spectrophotometer coupled to a microcomputer via a RS232 interface (De Meester *et al.*, 1987). In most cases the time courses remained first-order, and the $k_{cat.}/K_m$ values were deduced from a direct analysis of the curves. When separate $k_{cat.}$ and K_m values could be obtained, they were computed from initial-rate measurements and fitting of those data to the Henri–Michaelis equation with the help of the ENZFITTER program (Leatherbarrow, 1987). Addition of D-alanine or D-phenylalanine to the reaction mixture sometimes increased the rate of disappearance of the thioester substrate, thus allowing the computation of an acceleration factor under initial-rate conditions.

The ratio of transpeptidation to hydrolysis (T/H) was determined after separation of the excess substrate and the products by h.p.l.c. using an ET250/8/4 Nucleosil-5C_{18} column (Macherey–Nagel). The chromatography was done as follows: eluent A was a 10 mM aqueous solution of sodium acetate adjusted to pH 3.0 with HCl, and eluent B was acetonitrile. The elution rate was 1 ml·min^{-1} and detection was performed at 235 nm. The standard hydrolysis product was authentic benzoyl-D-alanine. The standard transpeptidation product was obtained by a transpeptidation reaction catalysed by the *Streptomyces* R61 DD-peptidase using substrate S2d as donor and 10 mM-D-alanine as acceptor (Jamin *et al.*, 1991).

RESULTS AND DISCUSSION

Fig. 2(*a*) shows the time course of the utilization of substrate S2d by t-PBP3s of *Ent. hirae*. The reaction was started by adding the enzyme to a 1 mM solution of the substrate. After a few minutes, D-alanine was added, which resulted in a distinct increase in the rate of disappearance of the thioester. Finally, the addition of benzylpenicillin rapidly and completely inhibited the reaction. The transpeptidation product was quantified by h.p.l.c. (Fig. 2*b*). Similar results obtained with the other PBPs are summarized in Table 1. In all cases, the presence of the transpeptidation product

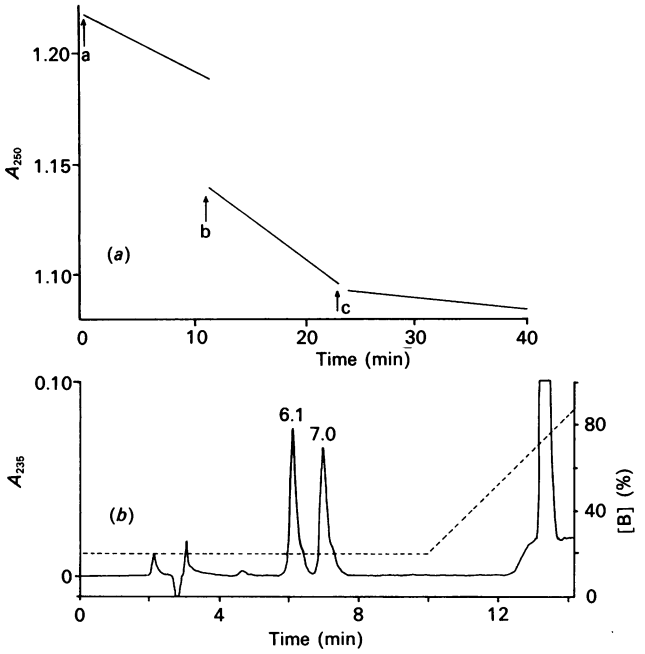

Fig. 2. Interaction between substrate S2d and the *Ent. hirae* t-PBP3s

(*a*) Time course (37 °C) of the disappearance of the substrate. At zero time (a), the enzyme (4 pmol) was added to a 1 mM solution of substrate in 475 μl of 10 mM-sodium phosphate, pH 7.0. After 11 min (b), D-alanine was added at a final concentration of 100 mM (the discontinuity is attributable to the dilution by the D-alanine solution). The reaction was finally stopped (c) by addition of 0.3 mM-benzylpenicillin. This demonstrated that the observed hydrolysis of the substrate was not a non-specific general base catalysis due to the various bases (histidine and lysine residues, for instance) present on the surface of the enzyme. The penicillin-binding site was thus specifically involved in the observed phenomena. The slow absorbance decrease ($0.5 \times 10^{-3} A \cdot min^{-1}$) recorded after the addition of benzylpenicillin was due to spontaneous hydrolysis of the donor substrate and was much lower than that due to the enzyme in the absence ($2.6 \times 10^{-3} A \cdot min^{-1}$) and the presence ($3.8 \times 10^{-3} A \cdot min^{-1}$) of D-alanine. The reaction could also be inhibited by a 10 min preincubation of the enzyme with 5 μM-cefotaxime, a specific reagent of PBP3s (Coyette *et al.*, 1983). (*b*) H.p.l.c. separation of substrate (S) and hydrolysis (H) and transpeptidation (T) products. The reaction mixture contained 25 mM-D-alanine, 1 mM-S2d and 20 pmol of enzyme in 500 μl of 10 mM-sodium phosphate, pH 7.0, and was incubated at 37 °C for 15 min. A 20 μl aliquot was injected into the column. The retention times were: transpeptidation product (benzoyl-D-alanyl-D-alanine), 6.1 min; hydrolysis product (benzoyl-D-alanine), 7.0 min; substrate S2d, 13.5 min.

was demonstrated by h.p.l.c. Most strikingly, with *E. coli* PBP3p, the hydrolysis reaction was nearly completely replaced by transpeptidation in the presence of only 5 mM-D-alanine.

Assuming that the interaction between the thioesters and the PBPs occurs according to a mechanism involving the formation of an acyl-enzyme, similar to that observed with the *Streptomyces* R61 DD-peptidase (Jamin *et al.*, 1991), the discussion of the present data can be based on a simple partitioning model where the acyl-enzyme, E-O-CO-CHR-NH-CO-C_6H_5, can undergo either hydrolysis or aminolysis by the acceptor. When acyl-enzyme accumulates at the steady state, the presence of the acceptor accelerates the rate of thioester utilization. This occurs with the *Ent. hirae* PBPs and *E. coli* PBP3p with substrate S2a. The absence of acceleration observed in the cases of the *S. pneumoniae* PBP and of *E. coli* PBP3p with substrate S2d can be most simply explained if no acyl-enzyme accumulates at the steady state. However, that simple model also predicts that the aminolysis to hydrolysis ratio (T/H) should remain proportional

Table 1. Kinetic characteristics of the hydrolysis and transfer reactions catalysed by HMM-PBPs

Abbreviation used: ND, not done. In all cases, the data were corrected for a small amount of spontaneous hydrolysis of the substrate.

PBP	Hydrolysis (H)				Transpeptidation (T)			
	Donor substrate	$k_{cat.}/K_m$ ($M^{-1}.s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	Donor substrate	Acceptor substrate	Acceleration factor	T/H
Ent. hirae t-PBP3s	S2a	< 20	—	—	S2d (1mM)	D-Alanine 10 mM	1.25	0.42
	S2d	3200*	6†	1.8†		50 mM	1.8	1.4
						100 mM	1.9	2.2
	S2e	3900*	ND	> 2		D-Phenylalanine (3 mM)	2.2	ND
					S2e (0.25 mM)	D-Alanine (10 mM)	ND	1.5
Ent. hirae t-PBP1d	S2d	1600†	ND	> 1	S2d (1 mM)	D-Alanine (50 mM)	2.2	0.67
Strep. pneumoniae PBP2Bc	S2a	< 20	—	—	S2d (1 mM)	D-Alanine 10 mM	1.0	0.24
	S2d	300	ND	> 1	S2e (0.25 mM)	10 mM	1.0	0.95
	S2e	1100	ND	> 2				
E. coli PBP3p	S2d	80‡	0.25†	3†	S2d (1.2 mM)	D-Alanine 5 mM	1.0	> 5
					S2a (1.2 mM)	10 mM	3.0	ND

* Based on a first-order analysis of a complete time course at $[S] \ll K_m$.
† Based on initial-rate measurements and for $k_{cat.}$ and K_m fitting of the values to the Henri–Michaelis equation.
‡ Ratio of the individual $k_{cat.}$ and K_m values.

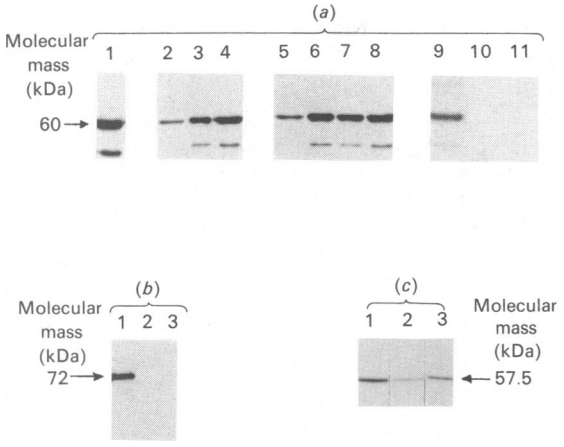

Fig. 3. Inhibition of the penicillin labelling of PBPs by the thioester substrates

(*a*) *E. coli* PBP3p. Tracks 1–8. Labelling was performed by incubating the purified PBP3p (2.5 pmol) with 1 μM-benzyl-[^{35}S]penicillin (1 mCi/μmol) for 1 min at 37 °C in 15 μl of 10 mM-sodium phosphate, pH 7.0, containing 250 mM-NaCl. Under those conditions, the PBP was 50 % saturated. Separation by SDS/PAGE and fluorography were performed as described by Bartholomé-De Belder *et al.* (1988). The fluorogram was exposed for 5 days. The various samples were first incubated for 2 min at 37 °C with substrates S2a and S2b and for 10 min with substrate S2d. Track 1, no substrate (reference); track 2, 20 mM-S2a; track 3, 20 mM-S2a and 100 mM-D-alanine; track 4, 10 mM-S2a; track 5, 20 mM-S2b; track 6, 20 mM-S2b + 100 mM-D-alanine; track 7, 10 mM-S2b; track 8, 5 mM-S2b. Tracks 9–11, the experiment was performed as described above, but [^{3}H]benzylpenicillin (10 mCi/μmol) was used and the fluorograms were exposed for 3 days. Track 9, no substrate; track 10, 10 mM-S2d; track 11, 10 mM-S2d + 50 mM-D-alanine. (*b*) *Streptococcus pneumoniae* PBP2Bc. The purified PBP2Bc (2.2 pmol) was incubated with 5 μM-[^{3}H]benzylpenicillin at 4 °C for 10 min in 15 μl of 10 mM-sodium phosphate, pH 7.0. The various samples were first incubated for 2 min at 37 °C with the following additions: track 1, no substrate; track 2, 20 mM-S2d; track 3, 20 mM-S2d + 50 mM-D-alanine. (*c*) *Ent. hirae* t-PBP3s. The purified t-PBP3s (2.5 pmol) was incubated for 2 min at 37 °C with 0.2 M-[^{3}H]-benzylpenicillin in 15 μl of 10 mM-sodium phosphate, pH 7.0. The various PBP samples were first incubated for 15 min at 37 °C with the following additions: track 1, no addition (this yielded less than 50 % of saturation of the PBP); track 2, 6.6 mM-S2d; track 3, 6.6 mM-S2d and 100 mM-D-alanine.

to the acceptor concentration (Frère, 1973) and that was not the case with *Ent. hirae* PBP3s. The performing of rigorous kinetic studies, necessary to propose a valid model for the reaction pathway, will be possible thanks to the simple substrate system described here.

It was also verified that the various substrates were able to inhibit the binding of radioactive benzylpenicillin to the PBPs. Such an inhibition, illustrated by Fig. 3, was consistently observed. Substrates S2c and S3d at a concentration of 20 mM also interfered with acylation of *E. coli* PBP3p by benzylpenicillin (results not shown).

In several cases, this inhibition was partly suppressed by the presence of the acceptor. Strikingly, the acceptor did not increase the labelling of the PBP by penicillin when it also did not accelerate the utilization of the donor, i.e. in the systems *S. pneumoniae* PBP2Bc + S2d and *E. coli* PBP3p + S2d. The experiments performed with *E. coli* PBP3p were particularly interesting in that respect: with substrate S2a, D-alanine partially restored acylation by benzylpenicillin (Fig. 3*a*, tracks 2 and 3) and increased the rate of donor utilization (Table 1). Conversely, with substrate S2d, neither acceleration nor restoration of labelling were observed (Fig. 3*a*, tracks 10 and 11; and Table 1). These results can also be explained on the basis of the simple model which implies that an acceleration of donor utilization induces a decrease in the steady-state level of acyl-enzyme. In consequence, more free enzyme is available for reacting with benzylpenicillin.

Finally, substrate S2d was found to be utilized in the presence of 50 mM-D-alanine by tryptic fragments of the HMM penicillin-resistant PBPs 5 and 3r of *Ent. hirae* (Jacques *et al.*, 1988; Piras *et al.*, 1990), but the reactions were very slow (preliminary determinations of the $k_{cat.}/K_m$ ratios yielded values of 10–40 $M^{-1} \cdot s^{-1}$).

In conclusion, the simple substrate systems that we describe here allow us to demonstrate, for the first time, the catalytic activities of several HMM PBPs. They will be invaluable when one wishes to perform rigorous analyses of the kinetic properties of these proteins and to build models accounting for these properties. We believe that the introduction of such substrates will also greatly facilitate the study of mutant enzymes obtained by protein engineering or discovered in penicillin-resistant strains. They represent a significant breakthrough in the study of the mechanism of action of β-lactam antibiotics.

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