Reaction of soluble penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus* with β -lactams and acyclic substrates: kinetics in homogeneous solution

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The kinetics of reaction of solubilized penicillin-binding protein 2a (sPBP2a) of methicillin-resistant *Staphylococcus aureus* with a variety of β -lactams and acyclic species was studied in homogeneous aqueous solution at 37 °C in 25 mM Hepes buffer, pH 7.0, containing 1 M NaCl. Under these conditions, but not at lower salt concentrations, protein precipitation did not occur either during or after the reaction. The reactions of β -lactams in general could be monitored by competition with a chromophoric β -lactam, nitrocefin, or directly in certain cases by protein fluorescence. Rate constants for reaction of a wide variety of β -lactams are reported. The interactions are characterized by a slow second-order acylation reaction followed by a slower deacylation. For example, the rate constants for benzylpenicillin

were $12 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3 \times 10^{-5} \text{ s}^{-1}$ respectively. The acylation is slow in comparison with those of normal non-resistant high-molecular-mass penicillin-binding proteins. sPBP2a also seemed to catalyse the slow hydrolysis of a variety of acyclic depsipeptides but not that of a D-Ala-D-Ala peptide. The reactions with certain depsipeptides also led to protein precipitation. These reactions were, however, not affected by prior blockage of the β -lactam-binding site by benzylpenicillin and thus might take place elsewhere on the enzyme. Two classes of potential transition-state analogue inhibitors, phosphonate monoesters and boronates, seemed to have little effect on the rate of reaction of sPBP2a with nitrocefin and therefore seem to have little affinity for the β -lactam-binding/D,D-peptidase site.

INTRODUCTION

β -Lactam antibiotics exert their action by inhibiting a class of enzymes, commonly referred to as the penicillin-binding proteins (PBPs), that catalyse the synthesis of the peptidoglycan of bacterial cell walls [1]. In general, whereas most low-molecular-mass PBPs possess only carboxypeptidase/transpeptidase activity, the high-molecular-mass PBPs can possess both transpeptidase and transglycosylase activities. PBP transpeptidase activity is inhibited by β -lactams because these compounds possess a structural similarity to natural substrate molecules (muropeptides) and form a serine-bound acyl-enzyme intermediate with the protein [1,2]. Although extensive use of β -lactam antibiotics has revolutionized the treatment of infectious disease over the past half-century, β -lactam-resistant forms of many pathogenic bacteria are a serious and growing threat to the efficacy of these antibiotics.

Resistance to β -lactam antibiotics can arise by a number of mechanisms, the best known being hydrolysis of the antibiotic by a β -lactamase. However, antibiotic resistance in many pathogenic bacteria can also be mediated by low-affinity variants of the high-molecular-mass PBPs arising by mutation, by deregulation of a normal high-affinity PBP or by synthesis of a low-affinity PBP *de novo* [3]. One of the best-known examples of PBP-mediated resistance is a low-affinity PBP in *Staphylococcus aureus*, PBP2a (or PBP2'), which is largely responsible for methicillin-resistance in these organisms (MRSA) [4]. MRSA strains continue to grow in the presence of high concentrations of β -lactam antibiotics owing to the expression of PBP2a (molecular mass 73.9 kDa) from the β -lactam-inducible *mecA* gene. The peptidoglycan is,

however, of abnormal muropeptide composition, particularly with respect to the low degree of cross-linking [5].

The *mecA* gene coding for PBP2a has been cloned and sequenced, showing PBP2a to contain structural motifs characteristic of a transpeptidase [6]. In addition, mutational analysis has demonstrated the functional presence of the conserved SXXX, SXN and KSG motifs of a normal transpeptidase in PBP2a [7]. No high-resolution crystal structure for any high-molecular-mass PBP with inherently low affinity for β -lactam antibiotics has yet been reported, although a 3.5 Å structure of PBP2x from *Streptomyces pneumoniae* has been published [8].

Although numerous biochemical studies have examined the interactions between PBPs and β -lactam antibiotics [9], few have resulted in detailed kinetic measurements and even fewer have involved the β -lactam-resistant high-molecular-mass PBPs. The membrane-bound nature of many high-molecular-mass PBPs has most likely impeded detailed kinetic studies. Wu et al. [7] first reported the construction of a water-soluble form of PBP2a (sPBP2a) by removal of the putative membrane anchor region. The subsequent purification and initial characterization of sPBP2a by both Roychoudhury et al. [10] and Frank et al. [11] have shown that homogeneous solutions of sPBP2a retain the ability to bind stoichiometrically to several β -lactams with the same apparent affinity as membrane-bound PBP2a. The affinities for several β -lactams also correlated well with their respective minimum inhibitory concentrations for methicillin-resistant *Staph. aureus* strain 27R [10,12].

Here we describe a detailed kinetic study of sPBP2a [11] in homogeneous solution. Rate constants were measured for its reaction with a number of penicillins and cephalosporins. In

Abbreviations used: MRSA, methicillin-resistant *Staphylococcus aureus*; PBP, penicillin-binding protein; sPBP2a, soluble penicillin-binding protein 2a.

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addition the interactions of sPBP2a with a variety of acyclic potential substrates and inhibitors were studied. The results presented here provide the first systematic overview of kinetic parameters for the interaction of a low-affinity, high-molecular-mass PBP with β -lactam antibiotics in homogeneous solution.

EXPERIMENTAL

Materials

The *mecA* (pET11d) T7 polymerase expression plasmid containing the coding sequence for a truncated form of the PBP2a protein from *Staph. aureus* was obtained from Merck Research Laboratories. Competent *Escherichia coli* BL21(DE3) cells were purchased from Novagen. The TSK-DEAE-5PW bulk ion-exchange resin was purchased from TosoHaas. The Superdex 200 HR 16/60 gel-filtration column was purchased from Pharmacia Biotech. Nitrocefin was purchased from UniPath, and pyridine-2-azo-*p*-dimethylaniline cephalosporin from Calbiochem. Benzylpenicillin, cephalothin, cephaloridine, ampicillin, methicillin and 6-aminopenicillanic acid were purchased from Sigma Chemical Co. and aztreonam was from ICN. Clavulanic acid was a gift from Beecham Pharmaceuticals; cefoxitin and *N*-formimidoylthienamycin were from Merck, Sharp and Dohme. Cefotaxime, 6 β -bromopenicillanic acid and moxalactam were gifts from Hoechst Pharmaceuticals, Leo Pharmaceuticals and Eli Lilly Co. respectively. All other substrates and inhibitors were from previous studies in this laboratory. Concentrated nitrocefin stock solutions were made by dissolving 0.5 mg of nitrocefin in 25 μ l of DMSO and 0.475 ml of 20 mM Mops buffer, pH 7.5. This solution was diluted into 25 mM Hepes buffer (pH 7)/1 M NaCl and filtered immediately before use. Stock solutions of all other antibiotics were made up in 25 mM Hepes (pH 7.0)/1 M NaCl and the pH was adjusted to 7.0 where necessary.

The expression and purification of recombinant sPBP2a were performed essentially as described by Frank et al. [11]. The final PBP2a protein solution was dialysed against 25 mM Hepes (pH 7)/1 M NaCl/0.01 % NaN₃ and stored at 4 °C. The concentration of sPBP2a was determined by measuring the absorbance at 280 nm and using a previously established molar absorption coefficient of 81 290 M⁻¹·cm⁻¹ ($A_{1\text{ cm}, 280, 1\%} = 1.1$ [11]). The final yield of sPBP2a was approx. 15 mg from 6.3 g of cell pellet and judged to be more than 95 % pure by SDS/PAGE and Coomassie Blue staining. Contamination of homogeneous sPBP2a by β -lactamase was examined by the turnover rate of benzylpenicillin and was determined to be insignificant.

Determination of kinetic parameters

Absorption spectra and spectrophotometric reaction rates were measured by means of a Perkin-Elmer Lambda 4B spectrophotometer. Spectrofluorometric measurements were performed with a Perkin-Elmer MPF 44A spectrofluorimeter. Unless stated otherwise all reactions were performed in a volume of 0.1 ml at 37 °C, in 25 mM Hepes buffer (pH 7.0)/1 M NaCl.

The rate of reaction between sPBP2a and nitrocefin was determined directly by monitoring the change in the absorbance of nitrocefin at 482 nm on acylation of sPBP2a [13]. Apparent first-order rate constants for acylation by nitrocefin were determined by fitting the change in absorbance with time to eqn. (1):

$$A = A_0 + \Delta\epsilon_n E_0 [1 - \exp(-k_n^* t)] + k_o N_0 \Delta\epsilon_n E_0 t \quad (1)$$

where A_0 is the initial absorbance at 482 nm, $\Delta\epsilon_n$ the change in molar absorption coefficient on nitrocefin hydrolysis

(16000 M⁻¹·cm⁻¹) [13], E_0 the initial enzyme concentration (generally 2.2 μ M), N_0 the initial nitrocefin concentration (0–500 μ M), k_n^* the (burst) pseudo-first-order rate constant for acylation of sPBP2a by nitrocefin, and k_o the second-order rate constant of a non-specific hydrolysis of nitrocefin in the presence of sPBP2a (see the Results section). Second-order rate constants (k_n) for acylation were calculated from a linear least-squares fit to a plot of the apparent first-order rate constants (k_n^*) against the concentration of nitrocefin.

Apparent first-order rate constants for acylation by other non-chromophoric β -lactams (k_i^*) were measured by direct competition with nitrocefin for sPBP2a. In this case, the change in absorbance at 482 nm with time was fitted to eqn. (2):

$$A = A_0 + \Delta\epsilon_n E_0 [k_n^* / (k_n^* + k_i^*)] \{1 - \exp[-(k_n^* + k_i^*) t]\} + k_o N_0 \Delta\epsilon_n E_0 t \quad (2)$$

where k_n^* is the pseudo-first-order rate constant for acylation by nitrocefin at the concentration (N_0) employed (generally 0.1 mM), k_i^* the pseudo-first-order rate constant for the reaction of sPBP2a with the competing β -lactam, E_0 the initial enzyme concentration (generally 2.2 μ M), and k_o the second-order rate constant of non-specific nitrocefin hydrolysis. Second-order rate constants (k_i) for acylation were calculated from a linear least-squares fit to a plot of the apparent first-order rate constants (k_i^*) against the concentration of β -lactam. All plots were linear for the concentrations of β -lactams used.

The slow deacylation of the acyl-enzyme complex formed between sPBP2a and benzylpenicillin was monitored by the reappearance of free enzyme as determined by its reaction with nitrocefin as described above. Equal concentrations of sPBP2a and benzylpenicillin (10 μ M) were incubated at 37 °C in 25 mM Hepes (pH 7.0)/1 M NaCl. At appropriate times an aliquot of the reaction was diluted into a reaction mixture containing 0.1 mM nitrocefin such that the final concentrations of sPBP2a and benzylpenicillin were each 2.2 μ M. The reaction with nitrocefin was subsequently followed as described above. The concentration of acyl-enzyme (EN) formed with nitrocefin was calculated by fitting the data to eqn. (1). The concentration of sPBP2a still acylated by benzylpenicillin (EI) was calculated by subtracting the concentration of EN from E_0 . The fraction of EI remaining in the reaction was plotted against time and compared with the expected concentration of EI by using a simulated model reaction (see Scheme 2) with the computer program KINSIM [14], where k_i was fixed at 12 M⁻¹·s⁻¹ (taken from the nitrocefin competition experiments described above) and the deacylation rate constant was varied from 0 to 5×10^{-5} s⁻¹ to obtain the best visual fit.

Fluorescence experiments:

The intrinsic fluorescence of sPBP2a was determined by exciting the samples at 288 nm and measuring the emission at 335 nm. The changes in fluorescence due to the interaction of sPBP2a with benzylpenicillin were measured by incubation of sPBP2a (1 μ M) with various concentrations of benzylpenicillin (0.05–0.2 mM). After the removal of excess benzylpenicillin by hydrolysis with 10 nM TEM-2 β -lactamase, the subsequent decrease in fluorescence was also measured over time. Apparent first-order rate constants for the increase and decreases in fluorescence were calculated by fitting the changes in the relative fluorescence intensities with time to simple exponential equations. The second-order rate constant for the increase in fluorescence was calculated from a least-squares linear fit to a plot of the apparent first-order rate constant against the concentration of benzylpenicillin.

RESULTS AND DISCUSSION

Preparation and properties of sPBP2a

The preparation of sPBP2a from inclusion bodies as described by Frank et al. [11] was successful in our hands, yielding 15 mg of essentially pure enzyme from 6.3 g of cell pellet. The pure protein is sparingly soluble in aqueous medium under conditions of low salt and low temperature. For example, we have observed precipitation of the protein at 4 °C at concentrations of NaCl of less than 0.15 M. At higher temperatures (up to 37 °C) and higher salt concentrations it seems soluble and active for long periods (at least 24 h). Some previous studies of this enzyme have also suggested that it becomes more susceptible to precipitation after acylation by β -lactams. For example, Roychoudhury et al. [15] have routinely observed the precipitation of sPBP2a at concentrations above 1 μ M by a variety of β -lactams in 0.05 M phosphate buffer including 0.1 M KCl at pH 8.0 and 37 °C. Indeed, they have described a chromogenic assay that relies on this fact. However, they and others have reacted sPBP2a with β -lactams under other conditions without protein precipitation [e.g. 10 mM Hepes/150 mM NaCl (pH 7.0) at room temperature] [11].

We performed initial kinetic studies on sPBP2a employing nitrocefin because of the large change in absorption spectrum on the opening of its β -lactam ring [13]. Roychoudhury et al. [15] have also employed nitrocefin to produce a chromophoric precipitate for their assay. First, we explored conditions under which sPBP2a acylation could be monitored in homogeneous solution without protein precipitation. On mixing nitrocefin (10–100 μ M) with sPBP2a (1–4 μ M) in 25 mM Hepes buffer in either 0.15 or 1 M NaCl and at either 25 or 37 °C, and monitoring nitrocefin β -lactam ring cleavage at 482 nm, we observed burst kinetics (Figure 1). The amplitude of the burst seemed to be stoichiometric with the enzyme concentration, and its rate increased with nitrocefin concentration. The burst was followed by a slow linear increase in absorbance whose slope increased linearly with enzyme and nitrocefin concentration. However, this slow reaction was not inhibited by benzylpenicillin at concentrations of the latter sufficient to preclude the nitrocefin burst (see below) and it thus seems unlikely to represent steady-state turnover of nitrocefin at the D,D-peptidase/ β -lactam-binding site. It might arise from a slow non-specific cleavage of nitrocefin by residues elsewhere on the protein. Nitrocefin is known to react with serum albumin, for example [13].

Figure 1 Absorbance changes at 482 nm on reaction of sPBP2a (2.3 μ M) with nitrocefin (0.1 mM) in the absence (solid line) and presence of 0.1 mM (broken line) or 1 mM (dotted line) benzylpenicillin

Figure 2 Variation with β -lactam concentration of pseudo-first-order rate constants of reaction of sPBP2a with nitrocefin (○) or with benzylpenicillin (●)

Scheme 1

Under the above-mentioned conditions, precipitation of protein was only observed immediately after the burst with the combination of low salt (0.15 M) and high temperature (37 °C). A sample of sPBP2a in 0.15 M salt after reaction with nitrocefin at 25 °C was also precipitated when the temperature was raised to 37 °C. Similar phenomena were observed with pyridine-2-azop-dimethylaniline cephalosporin, another chromophoric β -lactam. Replacement of NaCl with KCl (1 M) or Na_2SO_4 (0.5 M) did not significantly affect the kinetics of the nitrocefin reaction. There have been reports that the activities of some membrane-bound enzymes, including PBP1b of *E. coli* [16], when solubilized by truncation, are affected by detergents. In the present study, however, neither CHAPS or Triton X-100 at a concentration of 0.1% (w/v) had a significant effect on the nitrocefin kinetics. However, higher concentrations of CHAPS (1%, w/v) seemed to promote post-burst precipitation, e.g. in 0.15 M NaCl at 25 °C.

Acylation of sPBP2a by nitrocefin

In view of the above, all of the kinetic studies of sPBP2a described below were performed at pH 7.0 and 37 °C in 25 mM Hepes buffer containing 1 M NaCl. Under these conditions no further problems of protein precipitation were encountered. The higher temperature was chosen in preference to 25 °C simply on the basis of the higher rates of reaction, approx. 4-fold, observed at the former temperature. Application of eqn. (1) to the data from the nitrocefin burst kinetics (Figure 1) yielded values of the burst rate constant k_n^* , representing pseudo-first-order rate constants for the reaction of nitrocefin and sPBP2a. A plot of these against nitrocefin concentration was linear to 0.5 mM (Figure 2) with a slope of $53.2 \pm 3.2 \text{ M}^{-1} \cdot \text{s}^{-1}$. This can be interpreted in terms of k_n of Scheme 1, where N represents nitrocefin. The linear part of the absorbance change after the burst, as seen in Figure 1, can be interpreted as k_o of Scheme 1,

Scheme 2

Table 1 Second-order rate constants for reaction of sPBP2a with β -lactams

The reaction of nitrocefin was studied directly but all others were studied by competition with nitrocefin (see the text). The second entry for benzylpenicillin was obtained by the direct fluorescence method (see the text). Results are means $\pm$ S.D.

β -Lactam	k_i ($M^{-1} \cdot s^{-1}$)
Nitrocefin	53.2 ± 3.2
Benzylpenicillin	12.0 ± 0.6
	26.8 ± 1.8
Ampicillin	9.5 ± 1.1
Cephaloridine	4.1 ± 0.3
Cefoxitin	1.98 ± 0.15
Cephalothin	1.96 ± 0.15
<i>N</i> -Formimidoylthienamycin	0.89 ± 0.11
Cefotaxime	0.59 ± 0.07
<i>N,N'</i> -Ac ₂ -L-Lys-penicillanic acid	0.40 ± 0.02
Methicillin	0.39 ± 0.04
Moxalactam	0.29 ± 0.05
Clavulanic acid	0.093 ± 0.007
Aztreonam	0.070 ± 0.007
6-Aminopenicillanic acid	≤ 0.05
6-Bromopenicillanic acid	≤ 0.05

where E' represents the enzyme poised to catalyse the non-specific hydrolysis of N to the hydrolysis product P (see above). By interpreting the results in this way, a value for k_o of $2.30 \pm 0.34 M^{-1} \cdot s^{-1}$ was obtained.

By analogy with all precedent, the burst reaction of nitrocefin with sPBP2a is likely to represent acylation of the active-site serine residue by the β -lactam (Scheme 2), where $E \cdot N(I)$ represents the initial non-covalent complex and $EN(I)$ the acyl-enzyme. The linearity of the plot for nitrocefin in Figure 2 indicates that non-covalent binding of this β -lactam to sPBP2a is weak. In terms of Scheme 2, $K_s \geq 0.5$ mM and $k_2 \geq 0.014 s^{-1}$. Weak non-covalent association is typical of β -lactam/PBP interactions [9].

Acylation of sPBP2a by other β -lactams

The rate of acylation of sPBP2a by other β -lactams was determined in competition experiments with nitrocefin. Addition of sPBP2a to a mixture of nitrocefin and another β -lactam gave rise to the phenomenon shown in Figure 1 for benzylpenicillin. As the concentration of the second β -lactam was increased in successive experiments, the amplitude of the nitrocefin burst decreased and the pseudo-first-order burst rate constant increased. Application of eqn. (2) (derived from Scheme 1) to this result allowed the determination of pseudo-first-order rate constants k_i^* for the reaction of the β -lactam I with sPBP2a. A plot of k_i^* against the β -lactam concentration then yielded second-order rate constants k_i for the reaction of sPBP2a with the β -lactam. Such a plot for benzylpenicillin is shown in Figure 2.

Table 1 contains second-order rate constants k_i for the reaction of sPBP2a with a representative series of β -lactams. The dominant

Figure 3 Formation and decay of the acyl-enzyme (as a fraction of total enzyme) on reaction of sPBP2a (10 μ M) with benzylpenicillin (10 μ M)

The points are experimental and the line is calculated (see the text).

factors influencing reactivity seem to be chemical reactivity and the nature of the $C_{6(7)}$ side chain, as they typically are in the effectiveness of β -lactams as D,D-peptidase inhibitors and antibiotics [17]. The compounds most reactive with sPBP2a have an arylmethylacetamido side chain (nitrocefin, benzylpenicillin, ampicillin, cephaloridine, cefoxitin and cephalothin). Those that do not (*N*-formimidoylthienamycin, *N,N'*-diacetyl-L-lysyl-penicillanic acid, clavulanic acid, 6-aminopenicillanic acid and 6-bromopenicillanic acid) are less reactive. The bulky side chains of methicillin and of the oximino-amides cefotaxime, moxalactam and aztreonam also lead to resistance (as would be expected in MRSA).

The order of reactivity of the bicyclic β -lactams seems to mirror the order of their minimum inhibitory concentrations against homogeneous MRSA [10,18]. The rate of binding of benzylpenicillin to sPBP2a seems to be very similar to that to the membrane-bound enzyme under similar conditions [18]. Others have also noted the similarity of affinity of β -lactams for the two forms of the enzyme [11,12]. Comparison of the rate constant for benzylpenicillin with those of non- β -lactam-resistant PBPs, for instance those of *E. coli* (as summarized by Frère and Joris [9]) and *Staph. aureus* [19], all as membrane-bound species, suggests that the value for sPBP2a is smaller by factors ranging from 10 to 10^3 . In contrast, the benzylpenicillin rate constants for other β -lactam-resistant PBPs, those of *Enterococcus hirae* PBP3^r, *Ent. hirae* R40 PBP5 and *Ent. faecium* PBP5fm are very similar to that obtained for sPBP2a in this study. All of these β -lactam-resistant enzymes are thought to have closely similar structures [20,21].

Deacylation of penicilloyl-sPBP2a

The acylation and deacylation of sPBP2a in the presence of benzylpenicillin was monitored by nitrocefin acylation as described in the Experimental section, with the results shown in Figure 3. The best visual fit by simulation to these results gave a deacylation rate constant for benzylpenicillin, k_3 (Scheme 2), of $2.7 \times 10^{-5} s^{-1}$.

This reaction with benzylpenicillin could also be followed directly by means of the changes in protein fluorescence, as also described in the previous section. The results of a typical experiment of this type are shown in Figure 4. The protein fluorescence at first increased in intensity and then fell more slowly. The pseudo-first-order rate constant for the first phase increased with benzylpenicillin concentration in a linear fashion, giving a second-order rate constant of $26.8 \pm 1.8 M^{-1} \cdot s^{-1}$. It

seems likely that this would represent the rate of acylation of sPBP2a by benzylpenicillin. It may be noticed, however, that this value is twice that obtained from the nitrocefin competition experiment (Table 1). The reasons for this difference are unknown but a slow and unreproducible decrease in fluorescence was sometimes observed after the initial fluorescence increase. This unknown process might have led to a suppression of the maximal fluorescence and thus apparently to a greater rate constant. The slow decrease mentioned above could not be the (even slower) deacylation reaction, which was observed by fluorescence after the destruction of excess benzylpenicillin by β -lactamase (see the Experimental section). The deacylation rate constant obtained in this way from the slow fluorescence decrease (Figure 4) was $(3.3 \pm 0.7) \times 10^{-5} \text{ s}^{-1}$, in good agreement with the value from the nitrocefin assay.

It should be noted that Roychoudhury et al. [10] also reported a fluorescence intensity increase and then a decrease at single time points (15 min) from the incubation of sPBP2a with phenoxymethylpenicillin. They seemed to believe that the decrease in fluorescence intensity ('quenching') was correlated with acylation by β -lactams, but it seems more likely from the present study that the acylation by the penicillin at least leads to an enhancement of fluorescence. We observed a similar increase in fluorescence on the addition of ampicillin (100 μM) to sPBP2a. In other PBPs, however, fluorescence quenching on β -lactam binding has been observed [22,23].

The deacylation rate determined here for sPBP2a seems typical of PBPs in general [9,19], i.e. the structural changes leading to slow acylation are not also reflected in the deacylation rates. However, it is possible, even likely, that deacylation occurs by the elimination/hydrolysis route rather than by direct hydrolysis [24].

Reactions with acyclic species

sPBP2a is a very weak catalyst of the hydrolysis of a variety of acyclic depsipeptides that have been found to be substrates of β -lactamases [25–27] and to some extent PBPs [28,29]. The sPBP2a results are shown in Table 2 for compounds 1–5. Also included are results for the 'penicillin-shaped' acylaziridine 6 [30] (Figure 5) and the peptide *N,N'*-diacetyl-L-lysyl-D-alanyl-D-alanine 7, the latter thought to imitate the C-terminus of the natural peptide substrate [24]. The rates given, which can be taken as upper limits to $k_{\text{cat}}/K_{\text{m}}$, are clearly quite small, smaller indeed than $k_{\text{cat}}/K_{\text{m}}$ values for turnover of these substrates by several other PBPs [23,28,29]. In contrast, particular combinations of substrate and PBP have also previously exhibited no apparent reaction [28,29]. There are at present insufficient results in this area for broad generalizations to be possible. The generally low reactivity in this case, however, presumably is correlated with the low reactivity of sPBP2a with β -lactams.

The results of Table 2 contain the suggestion of a correlation between the rate of the enzyme-catalysed reaction and the chemical reactivity of the substrate, particularly the esters, towards nucleophiles, as estimated by the second-order rate constants for alkaline hydrolysis k_{OH} . A similar trend might also be present in the results in Table 1, along with that of the side chain structure as discussed above, because nitrocefin is chemically the most reactive of the bicyclic β -lactams represented, and 6-aminopenicillanic acid is the least reactive. The peptide 7 is by far the least chemically reactive of the compounds in Table 1 and is not discernibly a substrate of sPBP2a. This reluctance of sPBP2a to catalyse the hydrolysis of what might be considered the closest analogue of its natural substrate is typical of high-

Figure 4 Fluorescence intensity (335 nm) changes as a function of time on reaction of sPBP2a (1.0 μM) with benzylpenicillin (0.1 mM) on irradiation at 288 nm

Table 2 Rates of hydrolysis of depsipeptides catalysed by sPBP2a

Variable v is the measured initial velocity at 1 mM substrate (after subtraction of the background rate) divided by the enzyme and initial substrate concentrations. Abbreviation: n.d., no detectable reaction. See Figure 5 for structures of substrates.

Substrate	v ($\text{M}^{-1} \text{s}^{-1}$)	k_{OH} ($\text{M}^{-1} \text{s}^{-1}$)	Reference
1	58	1680	[27]
6	7.5	8.0	[30]
2	4.0	310	[27]
3	0.77	26	[27]
4	0.2	0.9	[27]
5	n.d.	0.9	[27]
7	n.d.	$< 10^{-5}$	[39]

molecular-mass PBPs [31]. The molecular basis of this curious rejection is not understood.

Noteworthy also with respect to the depsipeptides was the observation that the turnover of 1 and 2 (both 1 mM) by sPBP2a (2 μM) led to protein precipitation (even in 1 M NaCl at 37 $^{\circ}\text{C}$) in 5 min and 1 h respectively. It might be that transient acyl-enzymes generated from these compounds are less stable with respect to protein conformation than the long-lived variety generated through reaction with β -lactams. This might have consequences for antibiotic design: the inhibition of β -lactamases by such a mechanism is well established [32].

The acyl-enzymes generated from the reaction of PBPs and acyclic substrates are commonly accessible to specific amine nucleophiles, leading to an enzyme-catalysed aminolysis reaction [24,28,33]. No acceleration of hydrolysis of the depsipeptides 2–4 (1 mM each) was, however, observed in the presence of D-phenylalanine (20 mM) or the putative *Staph. aureus*-specific nucleophiles triglycine (20 mM) and pentaglycine (5 mM). Presumably this reflects the inaccessibility of the acyl-enzyme to the nucleophile, a high K_{m} for these substrates ($> 1 \text{ mM}$) or a rate-determining acylation step. Pentaglycine (5 mM) also had no effect on the kinetics of the nitrocefin reaction.

A final observation helped to clarify the observations with the acyclic species above: the reactivity of 2, 4 and 6 with sPBP2a was not significantly affected by a prior incubation of the enzyme with benzylpenicillin (1 mM) until no further nitrocefin burst

Figure 5 Structures of compounds 1–6 and 8

reactivity remained. This suggests that the major site of reaction of 1–6 is not at the β -lactam-binding site but elsewhere in sPBP2a, perhaps at the site of non-specific nitrocefin turnover. Whether it is reaction at the latter or the former site that is primarily responsible for protein precipitation is not clear at present, although it was found that covalent acylation of the former with benzylpenicillin did not perturb the precipitation process in the presence of 1 and 2. The possibility of the slow hydrolysis of reactive small substrates at other sites on PBPs should be generally considered in studies of these enzymes.

Finally, two classes of transition-state analogue inhibitors were found to have little effect on sPBP2a. The phosphonate 8 (2 mM) (Figure 5) did not inhibit the nitrocefin reaction over 1 h. Similarly, boric acid (70 mM) and phenylboronic acid (10 mM) had little effect, signifying K_i values well in excess of 100 mM. Both of these classes of compounds are effective β -lactamase inhibitors [34,35] supposedly because they form tetrahedral analogues to the transition states of acyl transfer, specifically of depsipeptide substrates in the case of 8, at the active site. The weak activity of sPBP2a (and of high-molecular-mass PBPs in general) against small acyclic substrates presumably correlates with the ineffectiveness of these transition-state analogues.

General conclusions

Conditions have been established under which the kinetics of interaction of sPBP2a with β -lactams and potential substrates and inhibitors can be studied in homogeneous solution. The quantitatively determined weak reactivity of sPBP2a with both β -lactams and acyclic substrates is in agreement with expectations for a methicillin-resistant PBP. Transition-state analogue species designed from the latter substrates are unlikely to be effective inhibitors/antibiotics unless much more specifically designed.

This enzyme seems to have evolved to a point of fine-tuned compromise where resistance to β -lactams is at the maximum possible level that still allows just enough peptidase activity (under optimal and at present unknown conditions) to maintain cell wall integrity. It is likely that complete β -lactam resistance and sufficient D,D-peptidase activity are mutually exclusive. These

considerations suggest that sPBP2a underwent a significant period of evolutionary optimization in an unknown organism before its relatively recent acquisition by *Staph. aureus* [36,37]. As with many of the high-molecular-mass PBPs, the peptidase active site might only assume a reactive conformation in the presence of its natural (polymeric) substrate and/or specific environmental factors that influence the active site's structure, for example the cell membrane and auxiliary proteins [38]. A close study of this enzyme should, however, give insight into the molecular details of the competing demands of β -lactam resistance and peptidase activity.

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