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Active-site serine mutants of the *Streptomyces albus* G β-lactamase

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By using site-directed mutagenesis, the active-site serine residue of the *Streptomyces albus* G β-lactamase was substituted by alanine and cysteine. Both mutant enzymes were produced in *Streptomyces lividans* and purified to homogeneity. The cysteine β-lactamase exhibited a substrate-specificity profile distinct from that of the wild-type enzyme, and its $k_{cat.}/K_m$ values at pH 7 were never higher than 0.1% of that of the serine enzyme. Unlike the wild-type enzyme, the activity of the mutant increased at acidic pH values. Surprisingly, the alanine mutant exhibited a weak but specific activity for benzylpenicillin and ampicillin. In addition, a very small production of wild-type enzyme, probably due to mistranslation, was detected, but that activity could be selectively eliminated. Both mutant enzymes were nearly as thermostable as the wild-type.

INTRODUCTION

Active-site-serine penicillin-recognizing proteins form a superfamily of bacterial enzymes that comprises most of the known β-lactamases and target enzymes of β-lactam antibiotics, DD-peptidases, involved in cell-wall biosynthesis (Ghuysen *et al.*, 1989). All these proteins interact with penicillins and cephalosporins through the formation of an acyl-enzyme (E–S*) where the antibiotic is covalently bound to the active-site serine residue (Scheme 1). In contrast with the peptidases, β-lactamases generally catalyse the deacylation step very efficiently (high value of k_3), which yields an inactive product (P) and regenerates active enzyme. On the basis of their primary structures, three classes of active-site-serine β-lactamases, A, C and D, have been identified. Alignment of the numerous sequences of class A β-lactamases (Ambler, 1980; Ambler *et al.*, 1991) conventionally defines the active-site serine as residue 70 (ABL numbering). All penicillin-interactive proteins share a few regions of conserved residues (Joris *et al.*, 1988), the active-site serine residue being the only one for which the role has been clearly demonstrated. Indeed, radiolabelling performed with poor substrates or inactivators pointed out serine as the covalently labelled side chain (Knott-Hunziker *et al.*, 1979; Cohen & Pratt, 1980; Fisher *et al.*, 1981; De Meester *et al.*, 1987*a*). More recently trapping of the acyl-enzyme with good substrates was demonstrated (Christensen *et al.*, 1990). Modifications of the active-site serine residue were performed by site-directed mutagenesis on the RTEM and *Bacillus licheniformis* β-lactamases (Sigal *et al.*, 1982, 1984; Dalbadie-McFarland *et al.*, 1982; Imanaka *et al.*, 1989). With such mutant proteins, hydrolysis might hopefully become sufficiently slow to allow the detection of intermediate(s) on the reaction pathway. Hence the most appropriate changes to introduce appeared to be Ser-70→Ala (mutant $S^{70}A$) or Ser-70→Cys (mutant $S^{70}C$), as these would have a minimal impact on the active-site geometry. A cysteine side chain would retain some nucleophilic character. However, the activities of the modified enzymes obtained by replacing an active-site serine residue by cysteine exhibit wide variations depending on the substrate tested (Polgár & Bender, 1967; Neet *et al.*, 1968; Sigal *et al.*, 1984; Higaki *et al.*, 1989). With the use of *Streptomyces albus* G β-lactamase, these mutations were performed and kinetic characterization of the mutants was undertaken to evaluate the consequences of the mutations. Both mutant proteins were greatly affected, although they retained some activity towards several substrates.

MATERIALS AND METHODS

Materials

Enzymes for genetic engineering were purchased from Biolabs (Beverly, CA, U.S.A.) and Boehringer (Mannheim, Germany); [γ-[^{35}S]thio]dATP (1350 Ci/mmol) was from NEN (Boston, MA, U.S.A.); crude ovalbumin was a gift from Belovo (Bastogne, Belgium). The origins and the structures of the β-lactam antibiotics used in the present study are given in Matagne *et al.* (1990). The following strains were used: *Streptomyces lividans* TK24 (Hopwood *et al.*, 1983), which was from the John Innes Institute Collection, and *Escherichia coli* TG1.

The *Streptomyces* plasmid pIJ702 (Katz *et al.*, 1983) was modified by Dr. Altenbüchner (Regensburg, Germany) in order to remove its *Kpn*I and *Bam*HI sites. Plasmid pDML6, as prepared by Dehottay *et al.* (1986), was used as a source of the β-lactamase gene.

Oligonucleotides were obtained from Eurogentech (Liège, Belgium). The crude oligonucleotides were purified by electrophoresis on a 20% polyacrylamide gel and desalted by using a spun column (Maniatis *et al.*, 1982).

Production of mutant enzymes

Site-directed mutagenesis was performed in bacteriophage M13 by using the Amersham 'Oligonucleotide-directed muta-

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{–}S^* \xrightarrow{k_3} E + P$$

Scheme 1. Interaction of a β-lactamase or a DD-peptidase with a β-lactam antibiotic

E is the enzyme, S the β-lactam, ES the Henri–Michaelis complex, E–S* the acyl-enzyme and P the product of hydrolysis.

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genesis kit' with the following oligonucleotides: for $S^{70}A$, CGATGTGTG*CGGTGTTC (17-mer), and for $S^{70}C$, GTTCCCGATGTGTTG*C*GTGTCCAAGACG (29-mer). After the mutagenesis reaction, clones were selected and completely sequenced to check for the absence of unwanted side-reaction mutations, as described in Jacob *et al.* (1990*a*). DNA sequencing was carried out by the dideoxy chain-termination method with the USB Sequenase kit. Cloning and production of the enzymes were as described previously (Jacob *et al.*, 1990*a*). Recombinant DNA techniques were based on Hopwood *et al.* (1985) and Maniatis *et al.* (1982). Culture conditions were described by Erpicum *et al.* (1990). The recombinant *Streptomyces* plasmids were called pDML271 ($S^{70}C$) and pDML273 ($S^{70}A$). Purification of the mutant β-lactamases was conducted as in Jacob *et al.* (1990*a*). Since the β-lactamase activities were too low for routine assay of the fractions during the purification, the enzyme-containing fractions were detected by using SDS/PAGE with the wild-type protein as a molecular-mass standard. For the $S^{70}C$ mutant, 1 mM-dithiothreitol and 100 μM-EDTA were added throughout the purification procedure. The concentration of the pure enzymes (> 90%) was determined by u.v.-absorbance measurements by using an absorption coefficient at 280 nm of 33000 $M^{-1} \cdot cm^{-1}$ (A. Matagne & J.-M. Frère, unpublished work). The proteins were stored at −20 °C in 50 mM-sodium phosphate containing 5% (v/v) each of ethylene glycol and glycerol (standard buffer).

Characterization of the mutant β-lactamases

Thermal denaturation. Thermal inactivation of the enzymes was monitored by fluorescence measurements. The enzymes were incubated at a fixed temperature, and samples were regularly withdrawn to measure the residual fluorescence of the enzyme at 340 nm (excitation at 280 nm) on a Kontron SFM 23 spectrofluorimeter. The curve was linearized according to a single-exponential decay:

$$F_t - F_\infty = (F_0 - F_\infty) \cdot e^{-kt}$$

from which the half-inactivation time was deduced. With the wild-type enzyme similar half-inactivation times were found by measuring either the fluorescence decrease or the loss of activity.

Determination of the number of thiol groups in the protein. A 5 μM solution of β-lactamase was incubated in the presence of a large excess of 5,5′-dithiobis-(2-nitrobenzoic acid). The appearance of 2-nitro-5-thiobenzoate was quantified at 412 nm, by using an absorption coefficient of 13600 $M^{-1}\,cm^{-1}$ as determined with solutions of free cysteine at 5–50 μM concentrations as standards (Ellman, 1959). Since thiol groups were not accessible in the native structure (Dehottay *et al.*, 1987), 0.1% SDS (final concentration) was added. The determinations were performed similarly for the wild-type and mutant enzymes.

Active-site titration with β-iodo[^{3}H]penicillanate. The $S^{70}C$ enzyme was incubated with β-iodo[^{3}H]penicillanate (2.6 mCi/mmol) (De Meester *et al.*, 1985) in large molar excess for 3 h. Extensive dialysis was performed over 60 h against 3 × 1 l of 50 mM-sodium phosphate buffer, pH 7, to remove the excess of reagent. The residual radioactivity after dialysis of a control sample containing the same concentration of β-iodo[^{3}H]-penicillanate and devoid of enzyme was subtracted.

Determination of the kinetic parameters of the mutant enzymes

Measurements were performed on a Beckman DU8, a Uvikon 860 or an HP Vectra spectrophotometer coupled to microcomputers via RS232 interfaces. Enzyme and substrate were mixed in the thermostatically controlled (30 °C) cuvette and the absorbance was recorded at 482 nm for nitrocefin, at 260 nm for other cephalosporins and at 235 nm for penicillins except for oxacillin and methicillin (260 nm). Only initial-rate measurements were taken since the reactions were too slow to obtain complete time courses within a practical period of time. When possible, separate values for $k_{cat.}$ and K_m were computed from Hanes plots; otherwise only $k_{cat.}/K_m$ values were determined at $[S] \ll K_m$.

Competition experiments were performed with nitrocefin as the reporter substrate, and K_m values for the competitor substrates were determined as K_i values. Inactivation experiments were performed by mixing enzyme, substrate (100 μM-nitrocefin) and inactivator and analysing the time course of nitrocefin hydrolysis as described by De Meester *et al.* (1987*b*). All incubations were performed at 30 °C in standard buffer.

RESULTS AND DISCUSSION

Production of the mutant β-lactamases

After mutagenesis and sequencing, the *KpnI–PstI* fragments coding for the mutant β-lactamases were cloned into the previously described *Streptomyces* plasmid (Jacob *et al.*, 1990*a*) to replace the equivalent wild-type fragment. After transformation of *Streptomyces lividans* TK24 protoplasts, the recombinant plasmids pDML271 ($S^{70}A$) and pDML273 ($S^{70}C$) were purified, and the DNA fragments coding for the β-lactamase were released by restriction, recloned into bacteriophage M13 and resequenced in the region of the mutation. This procedure was designed to avoid the occurrence of a wild-type gene in the plasmid preparations. The presence of the mutations was thus confirmed in pDML271 and pDML273. Mutant β-lactamases were then produced in 3-litre 6-day cultures and purified as described in Jacob *et al.* (1990*a*). Their chromatographic behaviours strictly paralleled that of the wild-type enzyme. The enzyme preparations were more than 90% pure as estimated by SDS/PAGE. With the $S^{70}C$ mutant, 100 μM-EDTA was added in all purification steps to protect the newly introduced cysteine residue from the action of heavy metals.

The stability of the mutant enzymes did not differ significantly from that of the wild-type (Table 1).

$S^{70}A$ mutant β-lactamase

At the end of the purification, this mutant β-lactamase retained a slight hydrolysing activity ($k_{cat.}/K_m$ for nitrocefin = 50 $M^{-1} \cdot s^{-1}$). In order to decide whether this activity was intrinsic or was due to contamination by the wild-type protein, the enzyme preparation (30 μM) was treated with the mechanism-based inactivator β-iodopenicillanate at a concentration of 10 μM. This resulted in a decrease in $k_{cat.}/K_m$ for nitrocefin to 5 $M^{-1} \cdot s^{-1}$, thereby indicating that the enzyme preparation contained about one wild-type enzyme molecule for 50000 mutant β-lactamase molecules. A contamination of the same order of magnitude was also present in a second batch of enzyme purified from another single colony after a new protoplast transformation with the pDML273

Table 1. Half-times for thermal inactivation of the β-lactamases

	Half-times (min)	
	45 °C	50 °C
Wild-type enzyme	55*	5±2
$S^{70}C$ enzyme	35±5	6±2
$S^{70}A$ enzyme	25±5	4±1

* From Matagne *et al.* (1990) (measured by the residual activity).

Table 2. Kinetic parameters $k_{cat.}$ and K_m for the $S^{70}A$ β-lactamase

Values in parentheses are for the wild-type enzyme (Matagne *et al.*, 1990).

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ (% of wild-type)
Benzylpenicillin	0.25±0.02 (2800)	1.3±0.5 (1)	200±50* (2.8×10^6)	0.007
Ampicillin	0.13±0.1 (3400)	1.2±0.35 (0.65)	120±30* (6.1×10^6)	0.002
6-Aminopenicillanate			< 20* (3.7×10^6)	< 0.0005
Nitrocefin			5±2 (2.4×10^6)	0.0002
Cephaloridine			< 1 (0.62×10^6)	< 0.0002

* Possible lag in the hydrolysis curve.

plasmid, and it was treated in the same way. Since the mutant β-lactamase was devoid of the active-site serine residue, it could not be inactivated by β-iodopenicillanate. Therefore the excess β-iodopenicillanate was not dialysed out, in order to prevent re-activation of the wild-type β-lactamase, which occurred with a first-order rate constant of about 10^{-5} s^{-1}, as measured on a wild-type enzyme preparation. The presence of the wild-type contaminant could be explained by mistranslation of the alanine codon resulting in the synthesis of a few molecules containing the wild-type serine residue. Such a case of mistranslation was reported in a study where the active-site serine AGC codon of the RTEM1 β-lactamase was replaced by the GGC glycine codon (Toth *et al.*, 1988). This mutant contained 0.1% of wild-type contaminant. The authors interpreted their results as an A/G wobble with a $tRNA^{Ser}$ at the first position in the codon–anticodon interaction.

Since the contaminant could be selectively inactivated, the intrinsic activity of the $S^{70}A$ enzyme could still be studied. This observation emphasizes a real problem when dealing with point-mutant enzymes, particularly those with intrinsic activity that represents only a very small fraction of that of the wild-type protein. It is difficult to guarantee a 100%-pure mutant preparation, and it might not always be as easy as here to demonstrate and eliminate the contamination.

The β-iodopenicillanate-treated mutant enzyme preparation was used to determine kinetic parameters for the best substrates of the wild-type β-lactamase (Table 2). Non-enzymic hydrolysis of the various substrates (< 10–20% usually) was measured and substracted in all cases so that the initial rates represented typical Henri–Michaelis saturation kinetics.

The mutant enzyme retained a significant activity against two substrates, benzylpenicillin and ampicillin, but other good substrates of the wild-type enzyme (6-aminopenicillanate, nitrocefin, cephaloridin) were barely hydrolysed or not at all. A slight lag was observed in the hydrolysis time courses for both benzylpenicillin and ampicillin. The $k_{cat.}/K_m$ value for benzylpenicillin was measured at both pH 7 and 9, and it was markedly lower at the latter pH.

The specificity profile was thus significantly different from that of the S^{70} enzyme. That the mutant enzyme exhibited any activity indicated that other groups in the active site can somehow activate a water molecule to perform a direct attack on the β-lactam amide bond. Our results were similar to those obtained by Carter & Wells (1988) with subtilisin. In that case the decrease in the catalytic efficiency was mainly due to a drastically lowered $k_{cat.}$ value while K_m was only slightly modified. Conversely, the same mutation in the *Bacillus licheniformis* enzyme resulted in a

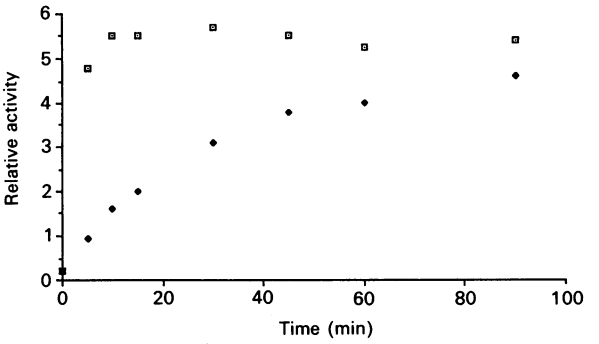


Fig. 1. Time course of the activation of the $S^{70}C$ β-lactamase on addition of 2-mercaptoethanol

□, 2 mM-2-Mercaptoethanol; ◆, 100 μM-2-mercaptoethanol.

complete loss of activity, although the β-lactamase appeared to bind benzylpenicillin reversibly but exhibited a strongly decreased affinity (Imanaka *et al.*, 1989). At present, this difference cannot be properly explained.

Preliminary crystallographic data indicate that the three-dimensional structure of the $S^{70}A$ was nearly identical with that of the wild-type: at the current level of resolution, all diffraction patterns were identical. The only difference between these two proteins is therefore the replacement of the serine $-CH_2OH$ group by the alanine $-CH_3$ group. Nevertheless, no complexes could be detected by X-ray diffraction after co-crystallization of the mutant with different substrates, or diffusion of substrates into the preformed crystal.

$S^{70}C$ mutant β-lactamase

Four free thiol groups were expected per molecule of mutant protein since the wild-type enzyme already contained three free thiol groups and no disulphide bond (Dehottay *et al.*, 1987). In the presence of SDS, 2.2±0.2 thiol groups were detected per mutant enzyme molecule, a result that might be explained by the formation of an intramolecular disulphide bridge involving the newly introduced cysteine residue. Moreover the activity of the enzyme increased more than 20-fold during a 10 min incubation with 2 mM-2-mercaptoethanol (Fig. 1). This latter molecule alone did not significantly degrade the β-lactam substrate in our experimental conditions.

A second purification was performed with the use of buffers containing both 100 μM-EDTA and 1 mM-dithiothreitol during

all steps. The specific activity of the purified second enzyme preparation was the same as that obtained after activation of the first one with 2-mercaptoethanol. Titration of the free thiol groups in the second preparation performed after rapid separation of the enzyme from the dithiothreitol by gel filtration on a small Sephadex G-25 column yielded a value of 3.7 ± 0.4 free thiol groups per enzyme molecule. Active-site titration of the $S^{70}C$ enzyme with a large excess of β-iodo[^{3}H]penicillanate indicated that at least 70% of the enzyme molecules could bind the inactivator. That only two thiol groups could be titrated before and four after reduction, whereas the same method detected three thiol groups in the wild-type β-lactamase, might suggest the formation of an intramolecular disulphide bridge. However, two of the three wild-type β-lactamase cysteine residues (Cys-123 and Cys-133) seem to be located too far from Cys-70 according to the three-dimensional structure (Dideberg *et al.*, 1987), if the folding of the mutant protein is identical with that of the wild-type. The last one, Cys-69, is right next to Cys-70. According to molecular modelling, a disulphide bridge between two adjacent cysteine residues would require the peptide bond between these two residues to be in a *cis* conformation, which is rather unlikely. At present we cannot provide any definite explanation for our observation.

The activity of the reduced mutant β-lactamase towards several substrates was determined (Table 3). The enzyme showed hardly any activity towards cephalosporins except nitrocefin, and K_m values were very high. The $S^{70}C$ mutant was therefore a considerably less active enzyme than the equivalent cysteine mutant of the RTEM β-lactamase (Sigal *et al.*, 1984). The specificity profile was not drastically altered, the best substrates, nitrocefin and ampicillin, also being efficiently hydrolysed by the wild-type β-lactamase. The high K_m values reported here are in agreement with the large increase in K_m towards nitrocefin observed for the RTEM mutant by Sigal *et al.* (1984). With several penicillins (penicillin G, methicillin), complex time courses were observed, indicating that their interaction with the mutant enzyme could be described not by a simple linear mechanism but probably by a branched pathway, implying several acyl-enzyme forms, as found by Kiener *et al.* (1980) in the interaction between 'type A substrates' (Citri *et al.*, 1976) and the β-lactamase I of *Bacillus cereus*. We could obtain no evidence, by monitoring the enzyme fluorescence during the reaction, of a conformation change.

Similarly to the behaviour of the wild-type enzyme, the $S^{70}C$ mutant was inactivated by β-iodopenicillanate. The interaction was studied on the basis of the model proposed by Frère *et al.* (1982), which was slightly modified to accommodate the results described above for the wild-type enzyme (Scheme 2). On incubation of the $S^{70}C$ β-lactamase (6 μM) with 1 mM-β-iodopenicillanate, a new rather wide absorbance band appeared between 290 and 370 nm, which could be explained by the sum of an enzyme-bound dihydrothiazine chromophore absorbing around 345 nm (Knap & Pratt, 1987) and the free dihydrothiazine absorbing at 305 nm in more-or-less equimolar proportions. After 4 h the absorbance at 345 nm was stabilized and corresponded to a nearly stoichiometric binding of the inactivator, while that at 305 nm slowly continued to increase. These results are in agreement with the model presented in Scheme 2, with $k_3 = k_4$ and a k_5 value of about 7×10^{-5} s^{-1}. In a second experiment, the enzyme activity towards a reporter substrate was monitored with β-iodopenicillanate concentrations ranging from 10 to 50 mM. From the apparent pseudo-first-order constants and ignoring the k_5 step, which was always at least 60-fold slower than the formation of (E–I)^i, the $(k_i)_{lim}$ and K_m values reported in Table 4 could be computed, showing that the most affected parameters were the acylation rate (k_2/K) and the k_3/k_4 ratio. The decrease in the latter ratio more probably reflects a decrease in k_3 than an increase in k_4, in agreement with the general decrease in enzyme efficiency towards all substrates.

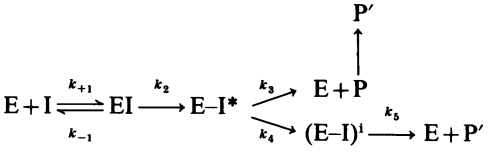
The pH-dependence of the $k_{cat.}/K_m$ value was determined in the pH range 3.5–10 with nitrocefin (Fig. 2). These results can be compared with those of Sigal *et al.* (1984) and Knap & Pratt (1989) with the same mutant of the RTEM β-lactamase. Quali-

Table 3. Kinetic parameters for the interaction of $S^{70}C$ β-lactamase with several antibiotics

Values in parentheses are standard deviations.

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ (% of wild-type)
6-Aminopenicillanate	0.06 (±0.006)	1.8 (±0.6)	35 (±3)	0.001
Benzylpenicillin	Non-Henri–Michaelis time course			
Ampicillin	1.2 (±0.3)	1.2 (±0.35)	1000* (±150)	0.015
Oxacillin			350 (±30)	0.05
Methicillin	Non-Henri–Michaelis time course			
Carbenicillin			20* (±3)	0.02
Nitrocefin			2000 (±120)	0.08
Cephaloridine		> 10	25 (±4)	0.004
Cephalothin		> 5	6 (±1)	0.0015
Cephalosporin C		> 10	≤ 5	≤ 0.012
Cefazolin		> 10	< 10	≤ 0.0015
Cephaloglycin		> 10	≤ 10	≤ 0.003
Cefuroxime		> 10	> 1	

* Possible lag in the hydrolysis time-course curve.



Scheme 2. Model for the interaction between β-iodopenicillanate and the wild-type and $S^{70}C$ mutant β-lactamases

E–I* is the acyl-enzyme, (E–I)^i a second more stable acyl-enzyme where the inactivator moiety has re-arranged into the dihydrothiazine chromophore, and P' the corresponding re-arranged product of P.

Table 4. Comparison of the kinetic parameters for the interaction with β-iodopenicillanate

	Wild-type*	Mutant ($S^{70}C$)
$(k_i)_{lim}$ (s^{-1})	0.047	0.04 ± 0.01
K_m (mM)	0.13	80 ± 20
$k_2/K = k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	180000	~ 1
k_3/k_4	515	~ 1
k_5 (s^{-1})	~ 10^{-5}	~ 7×10^{-5}

* From Frère *et al.* (1982).

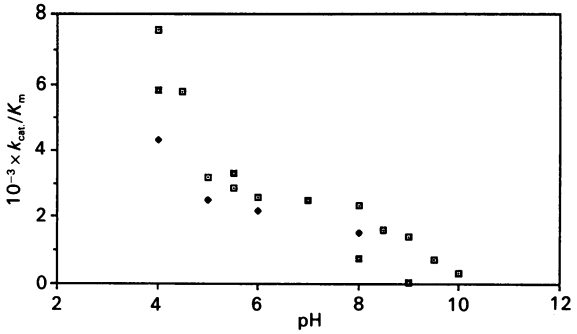


Fig. 2. pH-dependence of the $k_{cat.}/K_m$ values for nitrocefin and the k_i values for 10 mM-β-iodopenicillanate with the $S^{70}C$ β-lactamase

⊡, *S. albus* G enzyme+nitrocefin; ◆, *S. albus* G enzyme+β-iodopenicillanate; ■, RTEM enzyme+benzyl penicillin (from Sigal *et al.*, 1984). The following buffers were used: pH 4–5, sodium acetate; pH 6, sodium cacodylate/HCl; pH 7, sodium phosphate; pH 8–9, Tris/HCl; pH 10, sodium carbonate. All buffers were 50 mM and their conductivities were adjusted to 450 μS with NaCl. The $k_{cat.}/K_m$ values were determined by measuring initial rates at $[S] \ll K_m$. The rates of inactivation by β-iodopenicillanate were measured using the reporter substrate method. At pH 7, β-iodopenicillanate concentrations ranging from 10 to 50 mM were also used, yielding the individual values of $(k_i)_{lim}$ and K_m shown in Table 4. These results indicated a K_m value of 80 mM, and thus at 10 mM $(k_i)_{lim}/K_m$ could be approximated as $k_i/[\beta\text{-iodopenicillanate}]$. At pH 4, experiments were also performed with 5 mM-β-iodopenicillanate, yielding a k_i value amounting to about 50% of that observed with 10 mM inactivator. It could be safely assumed that all the k_i values for β-iodopenicillanate shown in this figure were determined at $[\beta\text{-iodopenicillanate}] \ll K_m$.

tatively, the results are rather similar. Hydrolysis of nitrocefin became faster at low pH, as mentioned by Sigal *et al.* (1984), and our $k_{cat.}/K_m$ profile for nitrocefin was similar to that obtained by Sigal *et al.* (1984) with benzylpenicillin (Fig. 2). The most remarkable feature was the sharp decrease in $k_{cat.}/K_m$ values when the pH was increased from 4 to 5.5. In our case the profile of the $(k_i)_{lim}/K_m$ values for β-iodopenicillanate was strikingly similar to that for the $k_{cat.}/K_m$ values for nitrocefin. This was not the case for the RTEM $S^{70}C$ mutant, for which Knap & Pratt (1989) obtained a simpler curve, suggesting the presence of a group exhibiting a pK_a of 6.5 and the acid form of which would be responsible for a rapid inactivation by β-bromopenicillanic acid. Such a pK_a could not be detected here. Our results could be reconciled with those of Knap & Pratt (1989) by assuming that in our case the whole curve is shifted towards the low pH range by at least 1.5 pH units. However, we believe the available results are too preliminary to allow the proposal of a mechanism that might explain all the experimental results. At the present time, the mechanism proposed by Knap & Pratt (1989) fails to explain the behaviour of the RTEM cysteine mutant with respect to benzylpenicillin.

Conclusion

Both active-site serine mutants retained a low but detectable activity, a result that was more surprising for the $S^{70}A$ β-lactamase. The activity towards cephalosporins was more affected than that towards penicillins, the only exception being nitrocefin with the $S^{70}C$ β-lactamase. These observations are in complete agreement with our previous reports (Jacob *et al.*, 1990*a*,*b*).

The enormous rate differences between the wild-type and $S^{70}A$ β-lactamase demonstrate the efficiency of the covalent catalysis in that type of enzyme.

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