A dramatic change in the rate-limiting step of β-lactam hydrolysis results from the substitution of the active-site serine residue by a cysteine in the class-C β-lactamase of *Enterobacter cloacae* 908R

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A cysteine residue has been substituted for the active-site serine of the class-C β-lactamase produced by *Enterobacter cloacae* 908R by site-directed mutagenesis. The modified protein exhibited drastically reduced $k_{cat.}/K_m$ values on all tested substrates. However, this decrease was due to increased K_m values with some substrates and to decreased $k_{cat.}$ values with others. These apparently contradictory results could be explained by a selective influence of the mutation on the first-order rate constant characteristic of the acylation step, a hypothesis which was confirmed by the absence of detectable acylenzyme accumulation with all the tested substrates, with the sole exception of cefoxitin.

INTRODUCTION

Class-C β-lactamases are chromosome-encoded proteins which can be responsible for high levels of resistance to β-lactam antibiotics in Gram-negative bacteria (Lindberg and Normark, 1986; Bush, 1989a,b). As shown by Scheme 1, their catalytic pathway involves the acylation of an essential serine residue, followed by the hydrolysis of the acylenzyme intermediate (Fisher et al., 1981; Knott-Hunziker et al., 1982; Joris et al., 1984).

β-Lactamases of classes A and D and penicillin-sensitive DD-peptidases hydrolyse β-lactams according to a similar mechanism, but with the latter the half-life of the acylenzyme is long and this results in an efficient inactivation of the enzyme. All these proteins seem to share similar three-dimensional structures, although their sequences can be very different (Joris et al., 1988; Herzberg and Moult, 1991 and references therein). The active sites of these enzymes do not appear to contain anything resembling the catalytic triad of active-site serine or thiol proteases.

No naturally occurring thiol β-lactamase, which would be equivalent to the cysteine proteases, has been found among the extremely large number of penicillin-hydrolysing enzymes described so far.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E.S \xrightarrow{k_{+2}} E\text{-}S^* \xrightarrow{k_{+3}} E + P$$

Scheme 1 Interaction of β-lactamase with a β-lactam antibiotic

E is the enzyme, S the β-lactam, E-S the Henri–Michaelis complex, E-S* the acyl-enzyme and P the product of hydrolysis. $K(=k_{-1}/k_{+1})$ is the dissociation constant of ES, and the two steady-state parameters are: $k_{cat} = (k_{+2}\cdot k_{+3})/(k_{+2}+k_{+3})$ and $K_m = (k_{+3}\cdot K')/(k_{+2}+k_{+3})$ where $K' = (k_{-1}+k_{+2})/k_{+1}$.

However, by site-directed mutagenesis, the active-site serine residue of the TEM and *Streptomyces albus* G β-lactamases (class A) have been replaced by cysteine residues, thus yielding artificial thiol β-lactamases (Sigal et al., 1984; Jacob et al., 1991). The modified TEM enzyme retained a larger proportion of the wild-type (WT) activity than the *S. albus* G enzyme but the $k_{cat.}/K_m$ values remained below 2 % of those of the WT. In both cases, the specificity profiles appeared to be altered. The same mutation has been performed in various penicillin-sensitive proteins (Broome-Smith et al., 1985; Houba-Hérin et al., 1985; Adachi et al., 1992; Hadonou et al., 1992): the R61 thiol DD-peptidase was completely devoid of activity and penicillin-binding properties. Similar results were obtained with *Escherichia coli* penicillin-binding proteins (PBPs) 2 and 3, but the modified PBP 3 seemed to retain a "slight" penicillin-binding capacity.

The *Enterobacter cloacae* 908R class-C β-lactamase has been extensively studied and its physico-chemical properties and kinetic parameters for various substrates have been established (Galleni et al., 1988a,b,c; Monnaie et al., 1992); in this paper we describe the replacement of the active-site serine by a cysteine residue in this enzyme and the properties of the resulting thiol β-lactamase.

MATERIALS AND METHODS

Strains and plasmids

The strains of *E. coli* K12 used in this work were SNO3 (*ampA1, ampC8, pyrB, recA, rpsL*) (Normark and Burman, 1977) and SNO302, an *ampD*⁻ derivative of SNO3 (Lindberg et al., 1987) for β-lactamase testing and production, FL01 (*recA* derivative of *E. coli* MM294) (Lindberg and Normark, 1987) for routine transformation and DNA preparation and TG1 [Δ(*lac–pro*), *supE, thi, hsdD5*/F′*traD36, proA*⁺*B*⁺, *lacI*^{q}, *kazZ*Δ*M15*] as a host strain for M13 phage growth.

The plasmids used were derivatives of pBR322 constructed by standard recombinant DNA techniques (Sambrook et al., 1989). They are represented in Figure 1.

Abbreviations used: dNTPs, dideoxynucleotide triphosphates; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulphonic acid; WT, wild-type; PBP, penicillin-binding protein; MIC, minimal inhibitory concentration; ssDNA, single-strand DNA.

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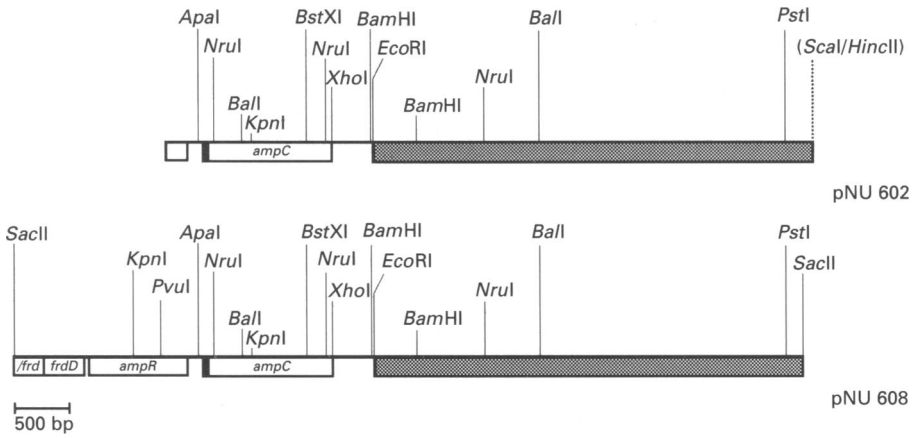

Figure 1 Restriction maps of plasmids pNU602 and pNU608

The darker area represents part of the pBR322 plasmid vector.

The procedure for *E. coli* transformation was described by Hanahan (1983).

Nucleic acid techniques

M13 phage was grown and single-strand DNA (ssDNA) prepared as described by Messing (1983). Mutagenesis was an adaptation of the method devised by Taylor et al. (1985), where T7 DNA polymerase (Sequenase) and T4 DNA polymerase were used for the first and second extensions respectively, with no change in the described buffers. This increased the efficiency of the method and reduced the overall duration of the procedure. Sequencing was done according to the dideoxy chain-termination method using the Sequenase DNA-sequencing kit (United States Biochemical, Cleveland, OH, U.S.A.).

Susceptibility testing

Minimal inhibitory concentrations (MICs) were determined on Mueller–Hinton agar (Difco Laboratories, Surrey, U.K.). The E-test strips (AB Biodisk, Sweden) were used to determine MICs using the flooding method, as described by the manufacturer, at 37 °C in air with incubation periods of 18–24 h.

Enzymes and chemicals

Benzylpenicillin was from Rhône–Poulenc (Paris, France), ampicillin was from Bristol Benelux S.A. (Brussels, Belgium), carbenicillin and cefuroxime were from Glaxo Group Research (Greenford, Middx., U.K.), cefotaxime was from Hoechst–Roussel (Romainville, France), cefoxitin was from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), cephalothin, cephalexin and cephaloridine were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), and 6-β-iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, U.K.). All these compounds were given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.).

T4 DNA ligase and polymerase were purchased from Boehringer–Mannheim Belgium (Brussels, Belgium), restriction endonucleases were obtained from Gibco BRL (Grand Island, NY, U.S.A.), New England Biolabs (Beverly, MA, U.S.A.) or Boehringer and used in the buffer supplied by the manufacturer. Dideoxyadenosine 5′-[α-^{35}S]triphosphate (10 mCi/ml) was purchased from Amersham International (Amersham, Bucks., U.K.) and dideoxycytidine 5′-[α-^{35}S]triphosphate was a product of Pharmacia (Uppsala, Sweden); other dideoxynucleotide triphosphates (dNTPs) were from Boehringer. The oligonucleotide was purchased from Symbicom (Umeå, Sweden). BSA, DL-phenyl-lactyl benzoylaminoacetate, iodoacetamide, 4,4′-dipyridyl disulphide, chloramphenicol and tetracycline were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Enzyme production and purification

Bacteria were grown for 26 h in 1-litre Erlenmeyer flasks each containing 500 ml of Terrific Broth medium (Sambrook et al., 1989) supplemented with 10 mg/l of tetracycline.

The enzyme was extracted and purified as described by Jacobs et al. (1992) with the sole difference that 2 mM dithiothreitol was added to all buffers except that used for elution of the enzyme from the CM-Sepharose column.

Routinely, protein concentrations were estimated by measuring the absorbance at 280 nm. For the pure β-lactamase a molar absorption coefficient of 83600 $M^{-1} \cdot cm^{-1}$ was calculated from the content of tryptophan and tyrosine residues on the basis of the equation given by Cantor and Schimmel (1980). The Bio-Rad protein assay kit (Bio-Rad Laboratories S.A., Belgium) was also utilized.

Determination of enzyme activity

β-Lactamase activity was determined by measuring the absorbance variation resulting from the hydrolysis of the substrate on Uvikon 860 (Kontron Instruments) or Beckman DU-8 spectrophotometers interfaced with microcomputers. All experiments were performed at 30 °C. Substrate hydrolysis was followed at 482 nm for nitrocefin, at 260 nm for other cephalosporins and at 235 nm for penicillins. Because of the low activity of the mutant enzyme, it was not possible to record complete reaction time-courses within reasonable periods of time. The initial-rate method was thus utilized and the steady-state parameters derived from Hanes–Woolf plots. When the K_m value was too high only the $k_{cat.}/K_m$ ratio was determined. Stopped-flow experiments were performed at 30 °C on a Bio-logic SFM3 apparatus. The kinetic parameters were derived from the initial rate of substrate

Table 1 MICs for the S64C- (pNU620, 660) and the WT-enzyme (pNU602, 608)-producing strains

MIC values are expressed in mg/l. Abbreviations used: n.d., not determined; Az, aztreonam; Ap, ampicillin; Pg, benzylpenicillin; Cef, cephalothin; Ctx, cefotaxime; Cfu, cefuroxime; Cox, cefoxitin; Ctz, ceftazidime; Tet, tetracycline.

	MIC (mg/l)								
Plasmid/strain	Az	Ap	Pg	Cef	Ctx	Cfu	Cox	Ctz	Tet
SNO3	0.064	n.d.	16	3	0.047	4	3	0.38	n.d.
pNU602/SNO3	0.19	24	> 256	> 256	0.50	6	4	1	96
pNU620/SNO3	0.064	2	16	3	0.047	4	3	0.38	96
SNO302	0.064	2	12	3	0.047	3	3	0.025	1.0
pNU608/SNO302	54	> 256	n.d.	> 256	256	> 256	128	> 256	96
pNU660/SNO302	0.5	2	8	8	0.094	6	0.094	0.75	96

hydrolysis or from competition experiments using nitrocefin as a reporter substrate. Derivation of initial-rate values from the collected datapoints and analysis of the inactivation time-courses (with iodoacetamide and β-iodopenicillanate) were done with homemade programs (De Meester et al., 1987). Fitting to the Hanes–Woolf equation and to the pH profiles was performed with the Enzfitter program (Leatherbarrow, 1987).

Activity versus pH studies

The following buffers were used, all with 200 mM NaCl added: 20 mM sodium acetate/acetic acid, pH 4.0–4.5; 20 mM sodium cacodylate/cacodylic acid, pH 5.0–6.5; 20 mM sodium phosphate, pH 6.0–7.5; 20 mM Hepes, pH 8.0; 20 mM Epps, pH 8.5; and 20 mM glycine/NaOH, pH 9.0. All buffers were adjusted to their pH value with either HCl or NaOH. The conductance (cell constant: 11.6/cm) of all buffers was 2 ± 0.2 mS. In some cases, 50 mg/l BSA was added to the buffers. All experiments were done at 30 °C.

pH dependence of thiol β-lactamase inactivation by iodoacetamide

Stock solutions of iodoacetamide were prepared in water immediately before use. The enzyme (100 nM final concn.) was added to a cuvette containing 100 μM nitrocefine and a known concentration of iodoacetamide (0.01–25 mM) in the appropriate buffer. The increase in absorbance at 482 nm was recorded and first-order rate constants derived from the datapoints. The second-order rate constants of inactivation (k_i) were obtained from the slope of the linear plot of the pseudo-first-order rate constant versus iodoacetamide concentrations.

Thermal inactivation

The enzymes (5 μM in 20 mM sodium phosphate, pH 7.0/200 mM NaCl) were incubated in a water-bath at a fixed temperature, and samples were withdrawn after increasing periods of time and assayed by following the enzyme activity against 100 μM cephalexin for the WT enzyme and 50 μM nitrocefin for the S64C mutant.

RESULTS

Mutagenesis

Plasmids pNU602 (*ampC*) and pNU608 (*ampC* and *ampR*) were constructed by cloning the *Hin*cII/*Eco*RI and the *Sac*II/*Eco*RI fragments from pNU368 (Galleni et al., 1988a) into the *Sca*I/*Eco*RI sites of pBR322 and the *Sac*II/*Eco*RI sites of pNU78 (Olsson et al., 1982) respectively (see Figure 1).

A 400 bp *Apa*I/*Kpn*I DNA fragment encoding the N-terminal part of *ampC* was cloned into an M13mp19 phage modified by an *Apa*I linker which had been introduced previously into the *Hin*cII restriction site of the polylinker. This construct was used to provide ssDNA for the site-directed mutagenesis reactions.

The oligonucleotide TACTTATACAACCCAGC was used to change the serine codon (TCT) into cysteine (TGT). Five phage plaques resulting from the mutagenesis procedure were sequenced and all of them exhibited the expected mutation. One clone was selected and the entire mutated insert sequenced from phage M13 ssDNA to ensure that no other unwanted mutation was present and was subcloned into pNU602 to yield plasmid pNU620. Subsequently, an *Apa*I/*Xho*I DNA fragment from pNU620 was subcloned into pNU608 to give plasmid pNU660 which was transformed into *E. coli* SNO302 for enzyme production.

Enzyme production

The strain pNU660/*E. coli* SNO302 has a stably derepressed β-lactamase-overproduction phenotype resulting from the *ampR*, *ampD*$^-$ combination (Lindberg and Normark, 1987). The S64C mutant (230 mg) was thus purified from 15 litres of culture.

After the CM-Sepharose step, the preparation appeared to be at least 95% pure by SDS/PAGE. It was used as such for physical and kinetic studies.

The β-lactamase activity totally disappeared upon addition of an excess of 4,4′-dipyridyl disulphide, with a concomitant increase in absorbance at 324 nm, which is characteristic of the reaction of thiols with this compound (Grasseti and Murray, 1967).

Enzyme stability

The half-life of the WT enzyme was 18 min at 60 °C whereas the S64C enzyme was slightly more stable, exhibiting half-lives of 12 and 100 min at 65 °C and 60 °C respectively. A Selwyn test (Selwyn, 1965) performed with nitrocefin at pH 4.5 and cephaloridine at pH 9.0 showed that the enzyme was stable ($t_{\frac{1}{2}} > 20$ min) over the whole pH range (30 °C, results not shown).

MIC values and steady-state parameters

Table 1 shows that production of the S64C enzyme did not increase the MICs at the highest level of β-lactamase production, i.e. in the pNU660/SNO302 case. The resistance to cefoxitin of a mutant-producing strain was even lower than that of the parent

Table 2 Steady-state parameters (pH 7.0, 30 °C) for the S64C mutant enzyme and their relative values compared with the WT enzyme

Substrate	$k_{cat.}$ (s^{-1})	% of WT value	K_m (μM)	K_m^{S64C}/K_m^{WT}	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	% of WT value
Benzylpenicillin	11 ± 1	60	2350 ± 210	4700	5900 ± 700	0.02
Ampicillin	0.10 ± 0.01	19	380 ± 40	950	250 ± 50	0.02
Carbenicillin	0.050 ± 0.004	1250	200 ± 20	20000	250 ± 20	0.07
Cefotaxime	0.023 ± 0.003	230	70 ± 15	7000	330 ± 80	0.03
Nitrocefin	> 18	> 2.2	> 1000	> 40	17600 ± 700	0.05
Cephalothin	$7.3 \pm 0.5\ 10^{-3}$	0.004	30 ± 10	3.3	240 ± 100	0.001
Cephaloridine	0.12 ± 0.01	0.02	105 ± 10	1.4	1190 ± 100	0.014
Cefoxitin	$6.5 \pm 0.5\ 10^{-4}$	6.5	1.0 ± 0.2	50	650 ± 50	0.06
DL-Phenyl-lactyl benzoylaminoacetate	–		–		20 ± 8	0.08

Table 3 Values of individual rate constants for WT and mutated (S64C) enzymes (pH 7.0, 30 °C)

Relative S.D. values are between 10 and 20%. The WT values for carbenicillin, ampicillin and benzylpenicillin are from Monnaie et al., 1992.

Substrate	K' (μM)		k_2 (s^{-1})		k_3 (s^{-1})	
	WT	S64C	WT	S64C	WT	S64C
Carbenicillin	15	200*	3	0.05	3×10^{-3}	> 0.05
Ampicillin	100	400	120	0.1	1	> 0.1
Benzylpenicillin	⩾ 5	800	> 150	11	20	> 11
Cephaloridine	> 75	100	⩾ 600	0.12	⩾ 600	> 0.12
Cefoxitin	40	> 13000	8	> 8	0.08†	0.0006

* Measured as a K_i value in competition experiments.
† The $k_{cat.}$ value from Galleni et al. (1988b) is taken as k_3.

strain without plasmid. This behaviour is not understood but might be correlated with the high induction potential of this particular β-lactam.

The values of the steady-state parameters are reported in Table 2. The enzyme did catalyse, however poorly, the hydrolysis of a representative set of penicillins and cephalosporins.

The mutation resulted in a drastic decrease of the $k_{cat.}/K_m$ parameter in all cases. It should be remembered that this represents a decrease of the second-order acylation rate constant k_{+2}/K'. However, when the individual parameters were considered, striking differences were observed. Indeed, most of the $k_{cat.}/K_m$ decrease could be attributed to an increase of K_m in the case of benzylpenicillin, ampicillin, carbenicillin and cefotaxime, and to a decrease of $k_{cat.}$ in the case of cephalothin and cephaloridine. These apparently contradictory results will be discussed below.

In the cases of carbenicillin and cefotaxime, a slight burst was observed but the progressive loss of activity did not amount to more than 70 and 30% of the initial rates respectively and the transition was rather slow ($t_{\frac{1}{2}}$ approx. 20 min). The values given in Table 2 were those observed under initial-rate conditions.

The enzyme retained some activity versus the depsipeptide DL-lactyl benzoylaminoacetate, for which the $k_{cat.}/K_m$ value was also less than 0.1% of the WT value.

The thiol β-lactamase was inactivated by 6-β-iodopenicillanic acid. No evidence for a branched pathway was found and the rearranged adduct exhibited an absorption maximum at 350 nm (325 nm for the WT), as observed with the TEM thiol β-lactamase (Knap and Pratt, 1987). The pseudo-first-order rate constant characterizing the inactivation was linear between 0.5 mM and 6 mM and a k_{+2}/K' value of $2.7 \pm 0.1\ M^{-1} \cdot s^{-1}$ was found, again considerably lower than that observed with the WT enzyme ($112 \pm 10\ M^{-1} \cdot s^{-1}$).

Occurrence of an acylenzyme

In contrast with what occurred with the other substrates (see below) accumulation of an acylenzyme could be visualized with cefoxitin, both by the reporter substrate method and by directly monitoring the disappearance of cefoxitin at 260 nm. The first method clearly indicated the accumulation of an inactive intermediate and the second showed this accumulation to be concomitant with the opening of the cefoxitin β-lactam ring (Figure 2). This demonstrated that the intermediate was the acylenzyme. Thus, with cefoxitin k_{+3} appeared to remain smaller than k_{+2}, although the k_{+2}/K' ratio decreased about 1500-fold and k_{+3} only 15-fold.

Conversely, when nitrocefin was used as a reporter substrate to monitor the binding of ampicillin, at concentrations varying from 3 to 15 times the K_m value, no time-dependent inhibition was observed, indicating that the steady state was established within the mixing dead-time (< 50 ms). In a parallel experiment, the establishment of the steady state was observed with cephaloridine as a substrate. With 2.5 mM cephaloridine (i.e. more than 20 times the K_m value), the steady state was also established within 60 ms (Figure 3). With carbenicillin, nearly complete acylation of the WT enzyme occurred at the steady state with saturating substrate concentrations ($k_{+2}/k_{+3} = 2500$; Monnaie et al., 1992) and the K_m value was quite low (10 nM). With the mutated enzyme, a K_i value of 200 μM was found and, when it

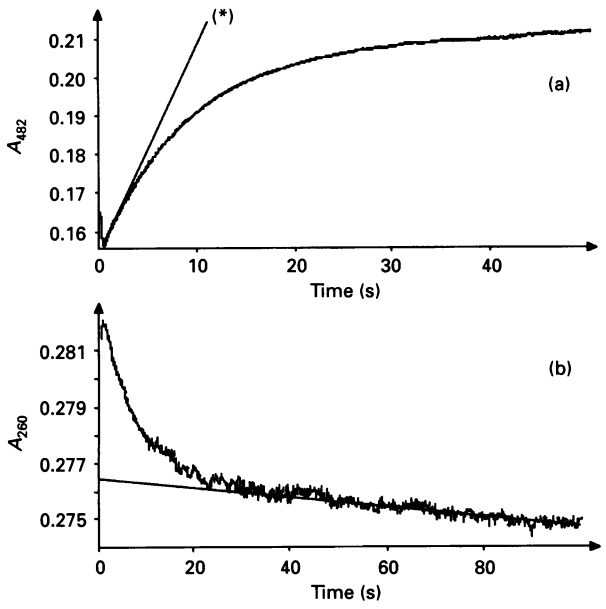

Figure 2 Rapid kinetic study of the interaction of cefoxitin with the thiol β-lactamase

(**a**) Inhibition curve of nitrocefin hydrolysis (100 μM, pH 7.0) in presence of 125 μM cefoxitin (1.65 μM enzyme). The straight line (*) represents the hydrolysis of nitrocefin in the absence of cefoxitin. (**b**) Hydrolysis of 125 μM cefotixin followed at 260 nm.

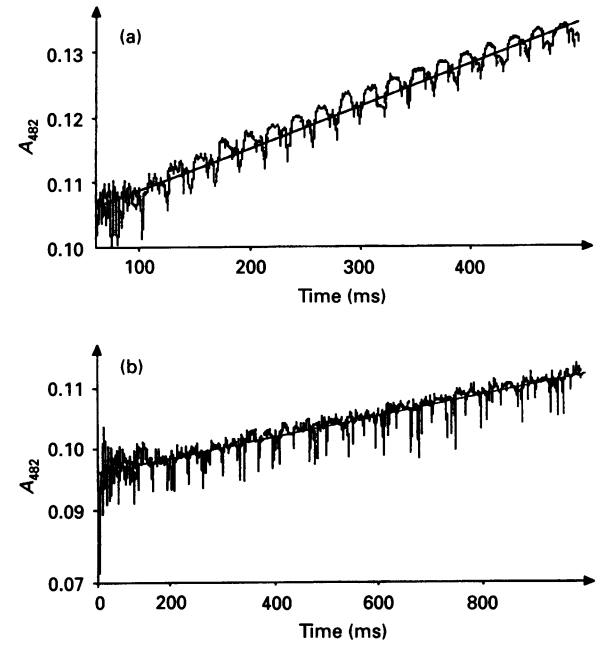

Figure 3 Rapid kinetic study of the interaction of ampicillin and cephaloridine with the thiol β-lactamase followed through the hydrolysis of 100 μM nitrocefin

(**a**) 2.5 mM ampicillin. (**b**) 2.5 mM cephaloridine.

was pre-incubated in the presence of 2 mM carbenicillin and subsequently diluted 100-fold into a 100 μM nitrocefin solution, no lag was observed in the hydrolysis curve of the reporter

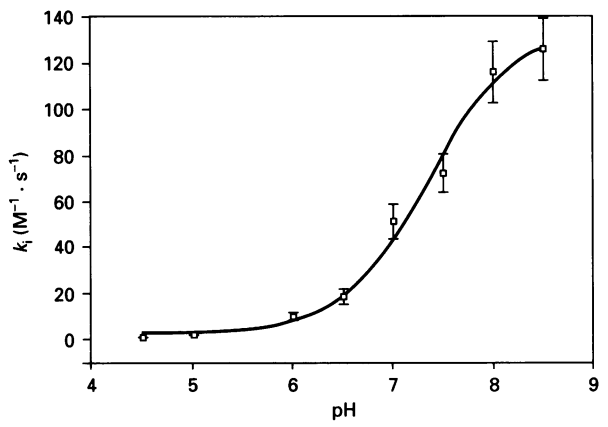

Figure 4 pH-dependence of thiol β-lactamase inactivation by iodoacetamide

For conditions, see the text. The error bars represent the error on the slope of the line ka = f([iodoacetamidel]). This slope supplied the value of k_i, the second-order rate constant of inactivation. The solid line was computed on the basis of the equation: $y = y_m + (y_M - y_m)/(1 + [H^+]/K)$ where K is the dissociation constant of the thiol group; y_m, y and y_M are the values of k_i at low (< 5), intermediate and high (> 9.3) pH values respectively.

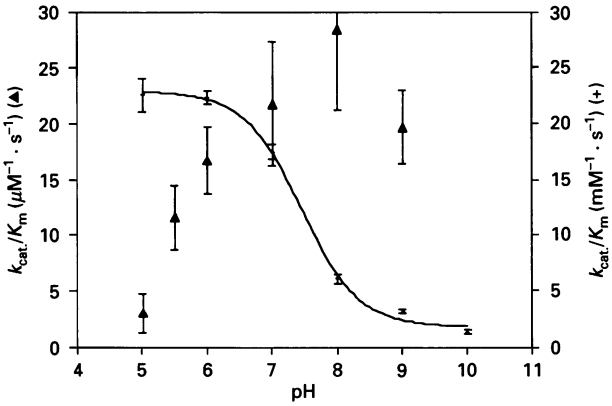

Figure 5 pH-dependence of the hydrolysis of nitrocefin ($k_{cat.}/K_m$) by the thiol β-lactamase (+) and the WT enzyme (▲)

The solid line characteristic of the former was computed on the basis of equation: $y = y_m + (y_M - y_m)/(1 + K/[H^+])$ where y_m, y and y_M are the values of $k_{cat.}/K_m$ at low (< 5), intermediate and high (> 9.5) pH values respectively. The error bars represent S.D. values (four determinations).

substrate, indicating the absence of the slow k_{+3} step easily detectable with the WT enzyme ($k_{+3} = 3 \times 10^{-3}$ s^{-1}). These results suggested that no acylenzyme accumulated with ampicillin, cephaloridine or carbenicillin.

Moreover, the addition of methanol, up to a 5 M concentration, failed to increase the rate of hydrolysis of benzylpenicillin and cephaloridine at saturating concentrations, in contrast with what was observed with the WT enzyme (D. Monnaie, unpublished work; Knott-Hunziker et al., 1982; Mazzela and Pratt, 1989).

Influence of the pH on the reaction rates

The rate of iodoacetamide inactivation was studied at various pH values (Figure 4). As expected the rate increased with pH and

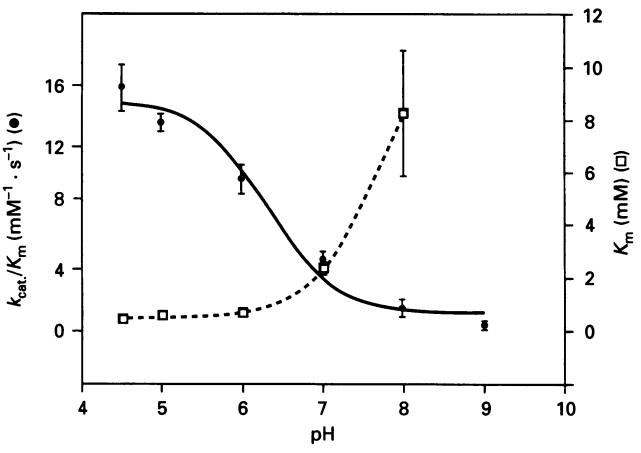

Figure 6 pH-dependence of the hydrolysis of benzylpenicillin ($k_{cat.}/K_m$; ●) with the thiol β-lactamase

The solid line was computed as in Figure 5. The variation of K_m is also shown (□), indicating that the decrease of $k_{cat.}/K_m$ is due to an increase of K_m. There is a 3-fold increase of $k_{cat.}$ between pH 6 and pH 9, but the large values of K_m at pH values $\geqslant 8$ made the determinations of the individual $k_{cat.}$ and K_m constants rather inaccurate. Indeed, $k_{cat.}$ values of 6.9 ± 0.4 and 22 ± 11 s^{-1} were found at pH 6.0 and pH 9.0 respectively. The error bars represent the errors on the parameters as obtained from the Hanes–Woolf plot with the help of the Enzfitter program.

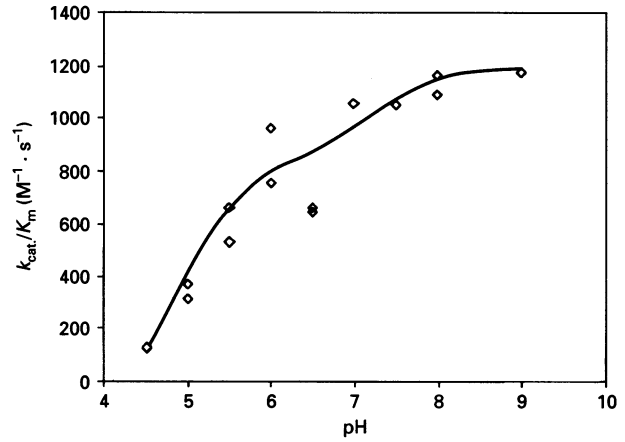

Figure 7 pH-dependence of the hydrolysis ($k_{cat.}/K_m$) of cephaloridine by the thiol β-lactamase

The points are experimental and the line was tentatively calculated for a double ionization system represented by the equation: $k_{cat.}/K_m = ([H^+]^2 y_{min.} + [H^+]Ka_1 y_{mid.} + Ka_1 Ka_2 y_{max.})/(Ka_1 Ka_2 + [H^+]Ka_1 + [H^+]^2)$. Where Ka_1 and Ka_2 are the first- and second-ionization constants and $y_{min.}$, $y_{mid.}$ and $y_{max.}$ are the values of $k_{cat.}/K_m$ at low (<4.5), intermediary (6–7) and high (> 9) pH values respectively (Fersht, 1985).

a pK value of 7.3 ± 0.2 was found for the pH-dependence of the inactivation process. Likewise, the $k_{cat.}/K_m$ value for nitrocefin exhibited a pH-dependence which could be fitted to a pK value of 7.4 ± 0.1, but in this case the rate decreased with increasing pH values (Figure 5). A similar dependence on pH was observed for benzylpenicillin (Figure 6) but the pK value was somewhat lower (6.3 ± 0.1). In this case the separate $k_{cat.}$ and K_m values which could be obtained indicated that the $k_{cat.}/K_m$ decrease at high pH values was entirely due to an increased K_m. Finally, with cephaloridine, the $k_{cat.}/K_m$ value exhibited a quite different pH-dependence, which did not seem to reflect the pK values determined with the three other compounds (Figure 7).

DISCUSSION

The thiol class-C β-lactamase which was obtained in this study exhibited widely decreased $k_{cat.}/K_m$ values for all tested substrates when compared with the WT enzyme. The MIC values confirmed the inefficiency of the mutant in an *in vivo* situation. Surprisingly, the MIC value for cefoxitin was even decreased when compared with that of the parent strain devoid of plasmid. This presently unexplained behaviour might be correlated to the high induction potency of this compound.

The $k_{cat.}/K_m$ decrease did not rest on variations of the same steady-state parameters in all cases. With benzylpenicillin, ampicillin, carbenicillin and cefotaxime very large K_m increases were observed accompanied by slight decreases or even increases of $k_{cat.}$ Conversely, with cephalothin and cephaloridine, the K_m values increased only 3.3-and 1.4-fold, while the $k_{cat.}$ values decreased dramatically. These apparently conflicting observations can be reconciled if one realizes that the compounds in the first group exhibit low to very low $k_{cat.}$ values with the WT enzyme, that these values have been shown to correspond to k_{+3} and that the k_{+2}/k_{+3} ratios are extremely large, up to 25000 for cefotaxime. No such ratio could be measured for cephalothin and cephaloridine, but if one assumes that for these two compounds the k_{+3} step is not severely rate-limiting (i.e. $k_{+2}/k_{+3} = 1$–3), all the results observed here can be explained by a considerable decrease of k_{+2}, possibly accompanied by an increase of k_{+3}. Indeed, when the k_{+2}/k_{+3} ratio is large, the decrease of k_{+2} mainly reflects on K_m, resulting in an increase of this parameter since $K_m = k_{+3} \cdot K'/(k_{+2} + k_{+3})$. Conversely, when the k_{+2}/k_{+3} ratio is lower, a large decrease of k_{+2} results in a moderate increase of K_m accompanied by a much more spectacular decrease of $k_{cat.}$, which corresponds to the situation observed with cephalothin and cephaloridine. For instance, assuming a k_{+2}/k_{+3} ratio of three for the WT enzyme with a given compound and that k_{+3} and K are not modified by the mutation, a 1000-fold decrease of k_{+2} will result in a 4-fold increase of K_m and a 250-fold decrease of $k_{cat.}$. The same 1000-fold decrease of k_{+2}, starting with a k_{+2}/k_{+3} ratio of 1000 just decreases the $k_{cat.}$ value by a factor of two, but increases K_m 500-fold.

The rapid kinetics experiments indicated that the steady state was reached within 0.1 s for one compound of each group (i.e. ampicillin and cephaloridine). Thus, one could not visualize the accumulation of acylenzyme, and it was quite likely that the measured $k_{cat.}$ values corresponded to k_{+2}. The only exception was cefoxitin, for which accumulation of the acylenzyme intermediate was still observed; however, it should be mentioned that even for this compound k_{+2}/K' decreased 1500-fold, while k_{+3} decreased only 15-fold. In most cases, the mutation thus transformed the catalytic properties of the enzyme, replacing a rate-limiting deacylation by a rate-limiting acylation. With some substrates, the deacylation rate was even increased. These results can be rationalized if one remembers that thiolesters are generally more sensitive to hydrolysis than oxoesters. In addition, the different size and bond angles of the sulphur atom would decrease the rate of acylation when compared with the serine enzyme where the geometry has been optimized for an oxygen nucleophile (it is worth remembering that the $k_{cat.}/K_m$ values of the WT enzyme are near the diffusion limit for several substrates). A similar specific decrease of the k_{+2} value might also explain some results obtained by Sigal et al. (1984) and Knap and Pratt

(1987) with the S70C mutant of the TEM β-lactamase (class A). Indeed, with benzylpencillin the k_{+2}/k_{+3} ratio is 1.9 (Christensen et al., 1990) for the WT enzyme and the mutation results in a drastic decrease of $k_{cat.}$ (85-fold) accompanied by a modest increase of K_m. Similarly, with cefoxitin, the k_{+2} value is 0.024 s^{-1} for the WT enzyme and the observed absence of interaction with the mutant probably reflects a negligible k_{+2} value.

As expected the anionic form of the thiol group exhibited a higher reactivity against iodoacetamide. By contrast the anion appeared to be less efficiently acylated by nitrocefin and benzylpenicillin than the neutral species. This might be explained by assuming a distortion of the active site due to the new negative charge, or by a repulsive interaction between this new charge and that of the substrate. The pH profile of the acylation by cephaloridine was completely different. This remains presently unexplained but might be correlated to the presence of a positive charge on the C′-3 side-chain of this compound.

In conclusion, the S64C mutation in a class-C β-lactamase drastically reduced the acylation rate while generally leaving the deacylation rate unaffected or even increased. Similar behaviour was observed with the K73R mutant of a class-A β-lactamase (Gibson et al., 1990), but in that case the values of k_{+2} and k_{+3} were not significantly different with the WT enzyme. To our knowledge, the mutation described here is the only one resulting in an inversion of the rate-limiting step in a β-lactamase mutant.

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REFERENCES

Adachi, H., Ishiguro, M., Imajoh, S., Ohta, T. and Matsuzawa, H. (1992) Biochemistry **31**, 430–437

Broome-Smith, J. K., Hedge, P. J. and Spratt, B. G. (1985) EMBO J. **4**, 231–235

Bush, K. (1989a) Antimicrob. Agents Chemother. **33**, 264–270

Bush, K. (1989b) Antimicrob. Agents Chemother. **33**, 271–276

Cantor, C. R. and Schimmel, P. R. (1980) Biophysical Chemistry, vol. 2, pp. 380–381, W. H. Freeman and Co., San Francisco

Christensen, H., Martin, M. T. and Waley, S. G. (1990) Biochem. J. **266**, 853–861

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frere, J. M. and Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403

Fersht, A. R. (1985) in Enzyme Structure and Mechanism (2nd edn.), p. 157, W. H. Freeman and Co., New York

Fisher, J., Charnas, R. L., Bradley, S. M. and Knowles, J. R. (1981) Biochemistry **20**, 2726–2731

Galleni, M., Lindberg, F., Normark, S., Cole, S., Honore, N., Joris, B. and Frere, J. M. (1988a) Biochem. J. **250**, 753–760

Galleni, M., Amicosante, G. and Frere, J. M. (1988b) Biochem. J. **255**, 123–129

Galleni, M. and Frere, J. M. (1988c) Biochem. J. **255**, 119–122

Gibson, R. M., Christensen, H. and Waley, S. G. (1990) Biochem. J. **272**, 613–619

Grasseti, D. R. and Murray, J. F., Jr. (1967) Arch. Biochem. Biophys. **119**, 41–49

Hadonou, A. M., Wilkin, J.-M., Varetto, L., Joris, B., Lamotte-Brasseur, J., Klein, D., Duez, C., Ghuysen, J.-M. and Frère, J.-M. (1992) Eur. J. Biochem. **207**, 97–102

Hanahan, D. (1983) J. Mol. Biol. **166**, 557–580

Herzberg, O. and Moult, J. (1991) Curr. Opinion Struct. Biol. **1**, 946–953

Houba-Hérin, N., Hara, H., Inouye, M. and Hirota, Y. (1985) Mol. Gen. Genet. **201**, 499–504

Jacob, F., Joris, B. and Frère, J.-M. (1991) Biochem. J. **277**, 647–652

Jacobs, C., Dubus, A., Monnaie, D., Normark, S. and Frère, J.-M. (1992) FEMS Microb. Lett. **92**, 95–100

Joris, B., Dusart, J., Frère, J.-M., Van Beeumen, J., Emanuel, E. L., Petursson, S., Gagnon, J. and Waley, S. G. (1984) Biochem. J. **223**, 271–274

Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C. and Knox, J. R. (1988) Biochem. J. **250**, 313–324

Knap, A. K. and Pratt, R. F. (1987) Biochem. J. **247**, 29–33

Knap, A. K. and Pratt, R. F. (1989) Proteins **6**, 316–323

Knap, A. K. and Pratt, R. F. (1991) Biochem. J. **273**, 85–91

Knott-Hunziker, V., Petursson, S., Waley, S. G., Jaurin, B. and Grundström, T. (1982) Biochem. J. **207**, 315–322

Leatherbarrow, R. J. (1987) Enzifitter: A Non-Linear Regression Data Analysis Program for the IBM PC/PS2, Biosoft, Elsevier

Lindberg, F. and Normark, S. (1986) Rev. Infect. Dis. **8**, 292–303

Lindberg, F. and Normark, S. (1987) J. Bacteriol. **169**, 758–763

Lindberg, F., Lindquist, S. and Normark, S. (1987) J. Bacteriol. **169**, 1923–1928

Mazzela, L. J. and Pratt, R. F. (1989) Biochem. J. **259**, 255–260

Messing, J. (1983) Methods Enzymol. **101**, part C, 20–78

Monnaie, D., Virden, R. and Frère, J.-M. (1992) FEBS, **306**, 108–112

Normark, S. and Burman, L. G. (1977) J. Bacteriol. **132**, 1–7

Olsson, O., Bergström, S. and Normark, S. (1982) EMBO J. **1**, 1411–1416

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (2nd edn.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Selwyn, M. J. (1965) Biochim. Biophys. Acta **105**, 193–195

Sigal, I. S., DeGrado, W. F., Thomas, B. J. and Petteway, S. R., Jr. (1984) J. Biol. Chem. **259**, 5237–5332

Taylor, J. W., Ott, J. and Eckstein, F. (1985) Nucleic. Acids Res. **13**, 8764–8785

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