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Role of the conserved amino acids of the 'SDN' loop (Ser^{130}, Asp^{131} and Asn^{132}) in a class A β-lactamase studied by site-directed mutagenesis

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Ser^{130}, Asp^{131} and Asn^{132} ('SDN') are highly conserved residues in class A β-lactamases forming one wall of the active-site cavity. All three residues of the SDN loop in *Streptomyces albus* G β-lactamase were modified by site-directed mutagenesis. The mutant proteins were expressed in *Streptomyces lividans*, purified from culture supernatants and their kinetic parameters were determined for several substrates. Ser^{130} was substituted by Asn, Ala and Gly. The first modification yielded an almost totally inactive protein, whereas the smaller-side-chain mutants (A and G) retained some activity, but were less stable than the wild-type enzyme. Ser^{130} might thus be involved in maintaining the structure of the active-site cavity. Mutations of Asp^{131} into Glu and Gly proved to be highly detrimental to enzyme stability, reflecting significant structural perturbations. Mutation of Asn^{132} into Ala resulted in a dramatically decreased enzymic activity (more than 100-fold) especially toward cephalosporin substrates, $k_{cat.}$ being the most affected parameter, which would indicate a role of Asn^{132} in transition-state stabilization rather than in ground-state binding. Comparison of the N132A and the previously described N132S mutant enzymes underline the importance of an H-bond-forming residue at position 132 for the catalytic process.

INTRODUCTION

The understanding of the molecular catalytic mechanism of β-lactamases remains a challenging task in view of the increasing clinical importance of β-lactam-resistant strains (Bush, 1989*a*,*b*,*c*). A very large number of different enzymes have been described so far, which can be classified in four main classes, A–D, on the basis of their primary structures. Among active-site-serine β-lactamases, class A enzymes are best characterized. Several tertiary structures have been refined and show a good superimposition of the active-site residues and secondary-structure elements (Herzberg & Moult, 1987; Dideberg *et al.*, 1987; Moews *et al.*, 1990); the alignment of the numerous sequences available reveals a few conserved regions (Ambler, 1980; Joris *et al.*, 1988; B. Joris, unpublished work), but the relationship between the important sequence variations and the significant discrepancies in the substrate profiles among class A enzymes remains largely unexplained (Matagne *et al.*, 1990).

It is assumed that the conserved active-site residues should play the same catalytic role in all class A β-lactamases, and site-directed mutagenesis experiments have been performed to unravel the mechanism, which remains so far relatively unknown. Except for the active serine residue, the role of which was assessed by labelling with mechanism-based inactivators (Cohen & Pratt, 1980; Fisher *et al.*, 1980) and site-directed mutagenesis (Dalbadie-McFarland *et al.*, 1982; Sigal *et al.*, 1984; Imanaka *et al.*, 1989), investigations were mainly conducted on charged residues. Mutation of Lys^{73} led to a very diminished enzymic activity (Madgwick & Waley, 1987; Gibson *et al.*, 1990), or even a loss of substrate binding (Imanaka *et al.*, 1989), but its function is still controversial. Mutation of Glu^{166} to Asp resulted in drastic decrease of both acylation and deacylation constants (Gibson *et al.*, 1990); it might act as a general base catalyst to facilitate proton transfer in the acylation and deacylation steps. Lys^{234} was shown to be important for substrate binding and transition-state stabilization (Ellerby *et al.*, 1990). Uncharged conserved residues have received very little attention so far, although they might play a critical role in substrate specificity (Hall & Knowles, 1976; Sougakoff *et al.*, 1988, 1989; Collatz *et al.*, 1989). It was shown in particular that mutation of Asn^{132} into Ser in the *Streptomyces albus* G β-lactamase drastically decreased the cephalosporinase activity of the enzyme (Jacob *et al.*, 1990). This Asn residue is part of the invariant 'SDN' region which forms one wall of the active-site cavity. According to three-dimensional models, both Ser^{130} and Asn^{132} side chains are pointing into the active-site and might thus be involved in the catalytic mechanism. In consequence, the side chain of Asn^{132} has been proposed as an H-bond partner of the acylamido side chain of the substrate (Herzberg & Moult, 1987). Although buried in the protein, Asp^{131} is also totally conserved, but its function remains undetermined. The surprising result obtained with Asn^{132} prompted us to investigate thoroughly all three positions by site-directed mutagenesis and kinetic studies on the mutant enzymes. In the light of those results, the function of these amino acids will be discussed. In the present paper we use Ambler's standard numbering of class A β-lactamases (ABL).

MATERIALS AND METHODS

Chemicals

Enzymes for genetic engineering were purchased from Biolabs (Beverly, MA, U.S.A.) and Boehringer (Mannheim, Germany); [^{35}S]dATP (1350 Ci/mmol) was from NEN (Boston, CT, U.S.A.); crude ovalbumin was a gift from Belovo (Bastogne, Belgium). The structures and the origin of the various penicillins and cephalosporins are given by Matagne *et al.* (1990). β-Iodopenicillanate was from Pfizer Central Research (Sandwich, Kent, U.K.). Thiostrepton was kindly given by Squibb (New Brunswick, NJ, U.S.A.). Dried media were obtained from Oxoid (Basingstoke, Hants., U.K.) and Difco Laboratories (Detroit, MI, U.S.A.).

Anion-exchange media and columns were purchased from Pharmacia (Uppsala, Sweden).

Strains, plasmids and growth conditions

The TG1 strain of *Escherichia coli* was used as a host for phage M13 and the *Streptomyces lividans* TK24 strain (Hopwood *et al.*, 1983) was used for enzyme expression and production. The *Streptomyces* plasmid plJ702 (Katz *et al.*, 1983) deleted from its endonuclease-*Kpn*I and *Bam* HI sites was a gift from Dr. J. Altenbüchner (Universität-Regensburg, Regensburg, Germany). The *Streptomyces albus* G β-lactamase gene was obtained from plasmid pDML6 (Dehottay *et al.*, 1986). For the expression of the mutant proteins, transformant *Streptomyces* colonies selected on thiostrepton R2YE agar were used to inoculate modified YEME medium (Erpicum *et al.*, 1990). Thiostrepton (25 mg/l) was added and Erlenmeyer flasks baffled with stainless-steel springs were used. The pH was adjusted daily to 7.2. High enzyme production was obtained after 6 days of growth.

Oligonucleotides

These were obtained from Eurogentech (Liège, Belgium). The crude oligonucleotide were purified by PAGE on a 20% polyacrylamide/8 M-urea gel and desalted by using a spun column (Maniatis *et al.*, 1982). The oligonucleotides used to introduce the mutations were the following: $Ser^{130}Ala$ ($S^{130}A$), TCC ATC ACC GCC *G*CC GAC AAC TGC G; $Ser^{130}ASN$ ($S^{130}N$), TCC ATC ACC GCC *AA*C GAC AAC TGC G; $Ser^{130}Gly$ ($S^{130}G$), C TCC ATC ACC GCC *GG*C GAC AAC TGC GCC GC; $Asp^{131}Glu$ ($D^{131}E$), CC ATC ACC GCC TCC GA*G* AAC TGC GCC GCC; $Asp^{131}Gly$ ($D^{131}G$), CC ATC ACC GCC TCC G*GC* AAC TGC GCC GCC; $Asn^{132}Ala$ ($N^{132}A$), CC GCC TCC GAC *GC*C TGC GCC GCC AA.

General methods

Recombinant DNA techniques were based on Maniatis *et al.* (1982). DNA sequencing was carried out by the dideoxy method using the USB Sequenase Kit. Specific *Streptomyces* DNA manipulations, such as protoplast preparation and transformation and plasmid extraction, were based on Hopwood *et al.* (1985).

Site-directed mutagenesis

The *Kpn*I–*Pst*I 1.1 kb fragment containing the *Streptomyces albus* G β-lactamase-coding sequence was cloned into M13tg131 for mutagenesis. The oligonucleotides were first assayed as sequencing primers in order to verify that their hybridization zone was correct. Owing to a very high (G+C) content in *Streptomyces* DNA (70%), secondary structures might occur in single-stranded templates and perturb the oligonucleotide hybridization. If necessary, the oligonucleotides were lengthened until they hybridized only on to the expected region. Site-directed mutagenesis was performed using the 'Oligonucleotide-directed *in vitro* mutagenesis' kit from Amersham based on the method of Taylor *et al.* (1985). After mutagenesis, single-stranded DNA was prepared from randomly picked transformants and mutant clones identified by sequencing using a primer which hybridized with a stretch of DNA located about 150 bp upstream to the mutated site. A mutant clone for each mutagenesis was sequenced entirely to confirm that no undesired additional mutation had been introduced. Sequencing was performed using the universal M13 sequencing primer and four internal oligonucleotides, priming every 250 bases.

Mutant β-lactamases: expression and purification

The expression vector was prepared by cloning the 1.4 kb DNA fragment containing the entire *Streptomyces albus* G β-lactamase gene isolated from pDML6 by restriction with *Pst*I and *Sst*I into the modified plJ702. The resulting plasmid was called pDML262. After mutagenesis and sequencing, the mutagenized inserts were released from their M13tg131 vector by restriction with *Kpn*I and *Pst*I and cloned into the corresponding sites of pDML262 to replace the wild-type fragment. The presence of the mutations in these final plasmids was confirmed by recloning the genes into M13mp19 and resequencing the zone of the expected mutation. Each of these plasmids was then used to transform *Streptomyces lividans* TK24 protoplasts. The mutant enzymes were purified from 3 litres of culture supernatant as described for the wild-type protein (Matagne *et al.*, 1990) with several modifications: desorption from DEAE-cellulose was performed batchwise with 0.25 M-NaCl; the filtration on Sephadex G-100 was omitted and a chromatography on a DEAE-Sephacel column in 50 mM-sodium phosphate buffer, pH 7, was performed after the chromatography on Q-Sepharose Fast Flow. After chromatography on DEAE-Sephacel, an almost homogeneous preparation was obtained. Chromatofocusing was performed as described for the wild-type enzyme (Matagne *et al.*, 1990) to reach a high level of purity, but this last step was detrimental for unstable mutant β-lactamases. For the active mutant proteins, the enzyme-containing fractions were detected using the nitrocefin assay, whereas fractions containing the nearly inactive $S^{130}N$ and $N^{132}A$ enzymes were detected by using SDS/PAGE with the wild-type β-lactamase as the molecular-mass standard. Enzyme homogeneity was demonstrated by the presence of a single band on SDS/PAGE. The enzymes were stored at -20 °C in 50 mM-sodium phosphate buffer, pH 7.0, containing 5% each of glycerol and ethylene glycol (hereafter called EG/G 5/5). Protein concentrations were routinely determined by absorbance measurements at 280 nm, with ϵ 33000 $M^{-1} \cdot cm^{-1}$ as obtained for the wild-type enzyme.

Determination of the kinetic parameters of the mutant enzymes

When possible a complete time curse of the hydrolysis of the antibiotic was recorded at 482 nm for nitrocefin, 260 nm for other cephalosporins, oxacillin and methicillin, and 235 nm for other penicillins. The values of the kinetic parameters K_m and $k_{cat.}$ were computed as described in De Meester *et al.* (1987*a*). When the hydrolysis was too slow to obtain a complete time course within 5–10 min, initials rates were measured and Hanes or Lineweaver–Burk plots were used to calculate K_m and $k_{cat.}$. When the K_m value was too high, only $k_{cat.}/K_m$ could be determined using a first-order time course at $[S] \ll K_m$. For very poor substrates, K_m values were obtained as K_i values in competition experiments using nitrocefin as a reporter substrate under initial-rate conditions. Inactivation experiments were performed by mixing enzyme, reporter substrate (100 μM-nitrocefin) and inactivator and analysing the time courses of nitrocefin hydrolysis as described by De Meester *et al.* (1987*a*). All incubations were performed at 30 °C in 50 mM-sodium phosphate buffer, pH 7, containing EG/G 5/5. Two spectrophotometers were used: a Beckman DU8 and a Uvikon 860 coupled to microcomputers via RS232 interfaces.

Stability measurements

The thermal stability of the active mutant proteins was determined by measuring the residual activity after increasing incubation periods at a fixed temperature as described by Matagne *et al.* (1990) for the wild-type enzyme. The buffer was 50 mM-sodium phosphate, pH 7, with EG/G 5/5. When the enzyme needed to be diluted, BSA (0.1 mg/ml) was added to the dilution buffer.

The rate of thermal denaturation for the nearly inactive $S^{130}N$ β-lactamase was monitored by fluorescence spectroscopy on a Kontron SFM23 spectrofluorimeter; the decrease of fluorescence emission at 340 nm (excitation at 280 nm) was measured after increasing incubation times at a fixed temperature. With the

wild-type enzyme both inactivation and fluorescence quenching yielded similar results.

Fractionation of *Streptomyces* cells

Three fractions were separated: the 'lysozyme-releasable' fraction, the membranes and the cytoplasm; fractionation was performed as described by Leyh-Bouille *et al.* (1977).

RESULTS

Expression of the mutant β-lactamases

All mutant β-lactamases but one were expressed in normal amounts (more than 50 mg/l for $S^{130}N$, $S^{130}A$, $N^{132}A$ and $D^{131}E$, and about 35 mg/l for $S^{130}G$), although usually lower than those described for the wild-type enzyme (Erpicum *et al.*, 1990). The time courses of production seemed to parallel those observed with the wild-type enzyme. In one case, however, (the $D^{131}G$ mutant) the production was drastically decreased; hardly any enzyme was detected throughout the culture, by using either an activity test or SDS/PAGE. This mutant β-lactamase was underexpressed; only the more sensitive Western-blotting and colony-immunoblotting techniques permitted the detection of a small level of enzyme. A transient expression occurred after 3 or 4 days of culture, but remained very low and disappeared over the following 2 days. This might indicate a largely decreased stability of the protein or an incorrect folding, leading to a much higher susceptibility to proteolysis.

Physico-chemical comparisons

The chromatographic behaviour of all S^{130} and N^{132} mutants was fairly parallel with that of the wild-type enzyme, although the chromatofocusing step proved to be detrimental to the less stable $S^{130}A$ β-lactamase. All purification yields were always significantly lower than those described for the wild-type enzyme (Matagne *et al.*, 1990), maybe because of a greater sensitivity of the mutant proteins to denaturation. The $D^{131}E$ behaved abnormally on ion-exchangers and could not be purified. For the $S^{130}A$, G, N and $N^{132}A$ mutants, the chromatofocusing patterns contained several bands which might correspond to several ragged *N*-termini as described for the wild-type enzyme.

Upon isoelectric focusing, the wild-type, $N^{132}S$ and $N^{132}A$ enzymes showed several bands in identical positions (pI from 4.5 to 5.5), whereas those of the $S^{130}A$ and $S^{130}N$ β-lactamases were slightly shifted towards more basic pH values. The $S^{130}A$ enzyme displayed an extra band at a pH of 5.8. C.d. measurements were performed on the wild-type, $S^{130}N$ and $N^{132}S$ β-lactamases with a Jobin–Yvon Dichrograph IV. Spectra were recorded between 200 and 260 nm with protein concentrations of 0.25–0.30 mg/ml, and they were nearly superimposable. The subtle structural differences that might be expected could therefore not be detected by c.d.

Under the conditions used for the wild-type enzyme, both $N^{132}S$ and $N^{132}A$ β-lactamases formed crystals, whereas the $S^{130}N$ and $S^{130}A$ enzymes failed to crystallize.

Thermal stability of the β-lactamases

The stability of the different enzymes was measured at pH 7 at various temperatures. The half-lives for mutant β-lactamases are shown in Table 1.

Although the enzyme concentration can influence the half-life (Matagne *et al.*, 1990), this effect was not such as to be worth taking into account here because of the large variations in the enzymic activities. All mutant β-lactamases were less stable than the wild-type, the $S^{130}A$ one being by far the most thermolabile. Residue N^{132} is unlikely to play any crucial role in enzyme stability, but the structure of the side chain in position 130 seemed to be a determining factor. Position 131 also appeared to be involved in protein stability.

Kinetic characterization of the mutant enzymes

The catalytic properties of the mutant β-lactamases were determined against a series of β-lactam antibiotics (penicillins and cephalosporins) chosen among the substrates studied with the wild-type enzyme (Matagne *et al.*, 1990). These antibiotics exhibited various structures and they ranged from very good to poor substrates of the wild-type enzyme. The interaction between the β-lactamase (E) and a substrate (S) is described by the model:

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

where ES is the non-covalent Michaelis complex and E-S* the acyl-enzyme.

Position 130

Three substitutions were performed for Ser^{130}: Ala, Asn and Gly. They were designed to probe for the role of this invariant serine residue in the cavity. Asn was somewhat bulkier, but retained H-bond-forming ability, whereas this possibility was lost with both Ala and Gly; the mutation into Ala introduced a hydrophobic side chain, and a complete absence of steric hindrance was expected with Gly.

The $S^{130}N$ β-lactamase showed a strongly decreased activity. The $k_{cat.}/K_m$ values for the best substrates were respectively 20 $M^{-1} \cdot s^{-1}$ (nitrocefin), < 100 $M^{-1} \cdot s^{-1}$ (ampicillin), 100 $M^{-1} \cdot s^{-1}$ (benzylpenicillin) and about 0 (6-aminopenicillanate). For benzylpenicillin, separate values of $k_{cat.}$ and K_m could be measured and were respectively 0.15 s^{-1} and 1.8 mM. Moreover that mutant seemed unable to bind β-iodopenicillanate covalently. A penicillin binding assay was performed (Nguyen-Distèche *et al.*, 1982) using 0.1 and 1 mM final benzyl[^{35}S]penicillin. The enzyme was significantly labelled, but that amounted to less than 10% of the total protein. This indicated a k_{+2}/k_{+3} ratio lower than 0.3, which led to the following individual values: 1.8 mM < $K = k_{-1}/k_{+1}$ < 2.3 mM, 0.15 $s^{-1} < k_{+2} < 0.2$ s^{-1}, $k_{+3} > 0.6$ s^{-1}. In contrast, both $S^{130}A$ and $S^{130}G$ β-lactamases retained a significant activity, as shown in Table 2. Separate values for $k_{cat.}$ and K_m could seldom be obtained (Table 3), since K_m values were usually high; the K_m values for penicillin substrates seemed particularly

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

S.D. values are within 20%. WT is wild type.

Lactamase	Temp. (°C)...	Half-life (min) 35	40	45	50
WT			⩾ 300	55	7(5*)
$N^{132}S$				18	
$N^{132}A$†				45	
$S^{130}A$†		80	9	1.5	
$S^{130}G$‡			75	9	
$S^{130}N$				20*	3*
$D^{131}E$§			45	5	

* Determined by fluorescence unfolding measurements.

† Incubated in concentrated solution (⩾ 50 μg/ml) because of the very low activity.

‡ Incubated in concentrated solutions (⩾ 50 μg/ml) and re-diluted for the measurement because of instability due to dilution.

§ Determined on crude culture supernatant (total concentration of proteins ≈ 5 mg/ml).

Table 2. $k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$) values for WT, $S^{130}A$ and $S^{130}G$ β-lactamases

S.D. values are within 10–20%. WT is wild-type; '%WT' is the percentage of the wild-type value.

Substrate	Lactamase...	$k_{cat.}/K_m$ value				
		WT*	$S^{130}A$	%WT	$S^{130}G$	%WT
6-Aminopenicillanate		3.7×10^6	3000	0.08	4200	0.1
Benzylpenicillin		2.8×10^6	70000	2.5	480000	17
Ampicillin		6.1×10^6	25000	0.4	140000	2.3
Carbenicillin		100000	900†	0.9	7800	7.8
Methicillin		52000‡	5500‡	10.5	18000‡	35
Oxacillin		630000	1000	0.16	3300	0.5
7-Aminocephalosporanate		170			$\leqslant 3$	$\leqslant 1.7$
Cephalexin		1700	< 1	< 0.1	< 1	< 0.1
Nitrocefin		2.4×10^6	200000	8.3	590000	25
Cephaloridin		620000	180	0.03	745	0.12
Cephalothin		370000	370	0.1	365	0.1
Cephalosporin C		40000	80	0.2	90	0.23
Cefazolin		560000			435	0.08
Cefuroxime		25000	$\leqslant 20$	$\leqslant 0.08$	6	0.02

* From Matagne *et al.* (1990).
† Burst.
‡ Lag.

increased for the $S^{130}G$ β-lactamase. With both enzymes, all cephalosporins but nitrocefin became poor substrates.

The time courses for the hydrolysis of carbenicillin obtained with the $S^{130}A$ mutant protein indicated substrate-induced inactivation ('burst'). This contrasted with the behaviour of the wild-type enzyme, which exhibited apparently normal time courses with that substrate, at least within the limits of the experimental method used (mixing dead time: 5–10 s).

The $S^{130}A$ β-lactamase seemed particularly unstable. During the last step of purification (chromatofocusing) the specific activity of the β-lactamase decreased, although other proteins were separated from the enzyme. Active-site titration with ^3H-labelled β-iodopenicillanic acid as performed in De Meester *et al.* (1987*b*) revealed that only 50% of the enzyme molecules in the final preparation remained capable of reacting with the inactivator. The active and inactive enzyme molecules could not be separated by chromatofocusing, which indicated rather similar conformations. In agreement with these observations the mutant protein exhibited a markedly decreased thermal stability (Table 1). In contrast, the $S^{130}G$ and $S^{130}N$ enzymes were both reasonably stable.

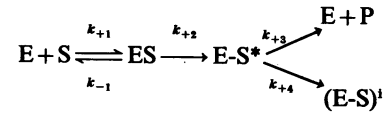
Table 3. K_m and $k_{cat.}$ values for several interactions

All S.D. values are within 10–20%. Most K_m values were estimated by competition experiments. Wild-type values are taken from Matagne *et al.* (1990).

β-Lactamase...	Wild-type		$S^{130}A$		$S^{130}G$	
Substrate	K_m (mM)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}$ (s^{-1})
6-Aminopencillanate	0.2	720	> 1	> 3	> 10	> 35
Benzylpenicillin	1	2800	1	70	> 5	> 2300
Ampicillin	0.65	3900	1.6	40	> 5	> 700
Oxacillin	0.43	270	> 1	> 1	> 10	> 32
Cephalothin	0.72	260	≈ 1	0.37	0.9	0.32
Cephalosporin C	4.5	170	10	0.75		

Interaction of $S^{130}A$ and $S^{130}G$ with β-iodopenicillanic acid

The suicide substrate β-iodopenicillanic acid was shown to interact with the wild-type enzyme according to a branched pathway:



where (E-S)' is an inactivated adduct in which S has rearranged (Frère *et al.*, 1982). Table 4 summarizes the results obtained for the wild-type, $S^{130}A$ and $S^{130}G$ β-lactamases.

Large differences were observed between the behaviour of the wild-type and mutant enzymes: the second-order rate constant for acylation was tremendously diminished probably due to both a decrease of k_{+2} and an increase of K'. The decrease of the k_{+3}/k_{+4} ratio could be explained by a decrease of the deacylation rate constant k_3 or an increase of k_{+4} but the available results do not allow to choose between these possibilities.

Position 131

Two mutations were attempted at this position, substitutions of Asp by Glu or Gly. The side chain of Asp^{131} points opposite to the active site and should not be expected to be involved in the actual catalytic mechanism itself; nevertheless, it is totally

Table 4. Parameter values for the interaction of the $S^{130}A$ and $S^{130}G$ lactamases with β-iodopenicillanate

Wild-type results are from Frère *et al.* (1982).

β-Lactamase	K_m (mM)	k_2/K' ($M^{-1} \cdot s^{-1}$)	k_3/k_4
Wild-type	0.13	180000	500
$S^{130}A$	> 10	40	45
$S^{130}G$	> 40	25	75

conserved among class A enzymes and probably fulfils a key function. The Asp-to-Glu mutation maintained the same negative charge and the possibility of retaining similar contacts within the protein, but the longer side chain should result in an adjustment of the SDN loop structure; the complete elimination of the side chain in the other substitution could be expected to induce some 'floppiness' of the SDN loop. Obviously both mutations severely affected the stability of the enzymes, especially $D^{131}G$. This β-lactamase was hardly detectable in the culture supernatant. A cell fractionation was attempted and all fractions submitted to SDS/PAGE and Western blotting in order to localize the enzyme. Hardly anything was detected in the culture supernatant and the 'lysozyme-releasable fraction', and nothing in the membrane and in the cytoplasm fractions. Segregation of the protein inside the cell due to an impaired secretion could thus be ruled out. The protein was probably not properly folded and rapidly proteolysed. This underexpression was not due to the choice of an infrequent codon for Gly, since GGC is much used in the gene coding for the *S. albus* G β-lactamase. Such low-level expression of unstable mutant proteins has been reported in several instances (Schultz & Richards, 1986). Consequently the mutant enzyme could not be produced and purified for further studies.

The $D^{131}E$ mutant was produced in reasonable amounts, but it suffered an unprecedented and surprising instability during purification. It lost ⩾ 99% of its activity upon contact with any anion-exchanger and exhibited abnormal tailing when eluted with an NaCl gradient. The loss of activity could not be attributed to the increased ionic strength of the elution buffer, or to non-specific adsorption of the enzyme on to the matrix. On the other hand, significantly more activity (20%) was recovered when the pH of the buffer used during the adsorption step was lowered to 5.5, a value much closer to the pI of the enzyme. Thus the loss of activity probably occurred during the interaction of the enzyme with the anion-exchanger groups, leading to an apparently irreversible conformation change. An attempt to obtain pure enzyme using non-denaturing preparative gel electrophoresis was also unsuccessful. Consequently the $D^{131}E$ mutant enzyme could not be further purified, and a few relative kinetic parameters ($V_{max.}/K_m$) were determined using crude culture supernatant (Table 5). A gross estimation of enzyme concentration was performed by using SDS/PAGE. In contrast with the S^{130} and N^{132} mutant β-lactamases, no significant specificity changes were noticed with the $D^{131}E$ protein, and a rather large proportion of the activity appeared to be conserved.

Table 5. K_m values and relative activity of the $D^{131}E$ mutant β-lactamase

The relative specificity is:

$$\left[\frac{(k_{cat.}/K_m)_{Antibiotic}}{(k_{cat.}/K_m)_{Benzylpenicillin}}\right] \times 100$$

The relative activity is:

$$\left[\frac{(k_{cat.}/K_m)_{D^{131}E}}{k_{cat.}/K_m)_{WT}}\right] \times 100$$

where WT is wild-type. The deviation arises from the estimation of enzyme concentration on SDS/PAGE.

Antibiotic substrate	K_m(mM)	Relative specificity: Wild-type	Relative specificity: $D^{131}E$	Relative activity of $D^{131}E$ (%)
6-Aminopenicillanate	2.3	132	100	4.5–9
Benzylpenicillin	5	100	100	6–12
Ampicillin	> 1	220	137	4–8
Carbenicillin		3.6	57	10–20
Oxacillin	> 5	22.5	17	5–10
7-Aminocephalosporanate	≈ 6	0.006	≈ 0	≈ 0
Cephaloridin	> 10	22	16	4.5–9
Nitrocefin		86	131	9.5–19
Cephaloridin	> 10	22	16	4.5–9
Cephalothin		13	46	2.2–4

Table 6. $k_{cat.}/K_m$ values for the interaction of the N^{132} mutant β-lactamases with a few antibiotics

S.D. values were within 10–20%. '%WT' means percentage of wild-type value.

Mutant β-lactamase...	$N^{132}A$				$N^{132}S$*	
Antibiotic substrate	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	$k_{cat.}/K_m$ (%WT)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	$k_{cat.}/K_m$ (%WT)
6-Aminopenicillanate	1.8	2	1100	0.03	100000	2.7
Benzylpenicillin	0.25	2	8000	0.3	1.5×10^6	53
Ampicillin	0.38	2	5250	0.09	10^6	16
Mecillinam			7000	0.27	2.5×10^6	100
Nitrocefin			20	0.0008	1200	0.05
Cephaloridin			< 5	< 0.0008	600	0.1
Cephalothin			< 5	< 0.0013	200	0.05

* From Jacob *et al.* (1990).

Position 132

Two mutations were performed at this position; the first one (Asn to Ser) modified the H-bond-forming side chain and was described in detail elsewhere (Jacob *et al.*, 1990). It yielded a new enzyme exhibiting a unique specificity profile. It had lost ⩾ 99% of its cephalosporinase activity while retaining a good penicillinase activity. The second substitution (Asn to Ala) was designed to eliminate totally the H-bond-forming capacity and the polarity of the side chain. Very few kinetic parameters could be determined with that latter mutant, since it was almost totally inactive (Table 6), even with the best substrates. It never exhibited more than 0.5% of the wild-type activity, the best substrates being the penicillins which were already good substrates of the wild-type enzyme: penicillin G, ampicillin, 6-aminopenicillanate and mecillinam. The increase in activation energy for acylation of the active serine by the substrate when replacing Asn by Ala could be estimated from:

$$\Delta(\Delta G^*) = -RT \ln[(k_{cat.}/K_m)_{mut.}/(k_{cat.}/K_m)_{WT}]$$

as 13.8–20.5 kJ (3.3–4.9 kcal)·mol^{-1}, depending on the substrate, underlining the importance of the H-bond-forming side chain of Asn^{132} for the catalytic process. When it could be determined, the more affected parameter was $k_{cat.}$ (1000- and 2000-fold decrease for penicillin G and ampicillin), whereas K_m was not drastically modified. An increase of activation energy can be attributed to ground-state stabilization or transition-state destabilization or both. However, since the K_m values were unchanged, the $\Delta(\Delta G^*)$ values mainly reflected transition-state destabilization.

DISCUSSION

Although no emphasis has been placed so far on the potential importance of the amino acids of the 'SDN' loop, all mutations performed in the present study clearly demonstrated the import-

ant role of this invariant region for the good functioning of the enzyme. So far no complex of a class A β-lactamase with a normal substrate has been available for crystallographic studies, and this makes it very difficult to attribute a definite role to any of the catalytic-site amino acids save Ser^{70}.

Position 130

Mutant $S^{130}N$ suffered the most drastic loss of activity. The three-dimensional structure shows that Ser^{130} is one of the closest neighbours of the active Ser^{70}. Indeed the substitution of Ser^{130} by a bulkier side chain residue seemed to result in steric hindrance. That demonstrated the need for a small residue at position 130. Accordingly the $S^{130}A$ and $S^{130}G$ mutant proteins, although sometimes severely impaired when compared with the wild-type enzyme, were about 1000-fold as active as the $S^{130}N$ β-lactamase. The $S^{130}A$ protein, however, showed a markedly decreased thermostability, whereas that of the $S^{130}G$ and $S^{130}N$ enzymes was not very different from that of the wild-type. Moews *et al.* (1990) have hypothesized that the corresponding residue in *Bacillus licheniformis* makes an internal H-bond with Lys^{234} (which belongs to the conserved 'KTG' motif), thus bridging the two protein domains at the hinge of which lies the active cavity. The presence of the hydrophobic side chain of Ala might destabilize the active-site cavity more than the complete absence of side chain. The better stability of the $S^{130}N$ mutant could be explained by the formation of alternative H-bonds with side chain(s) of the other domain, leading to a slightly modified, and consequently far less active, conformation around the catalytic site. These unstable ($S^{130}A$) or modified ($S^{130}N$) conformations would be reflected by slightly different isoelectrofocusing patterns and the inability of these proteins to crystallize under the same conditions as the wild-type.

Comparison of the $S^{130}A$ and $S^{130}G$ mutant enzymes showed that their respective specificities were parallel. The best substrates for the wild-type enzyme remained the best for both mutants: ampicillin, penicillin G and nitrocefin. Conversely, the catalytic activities versus cephaloridin, cephalothin and 6-aminopenicillanate, which were fair to good substrates for the wild-type, suffered a disproportionate decrease ($k_{cat.}/K_m$ values amounted to $\leqslant 0.1\%$ of those of the wild-type enzyme), due in part to an increased K_m for 6-aminopenicillanate. If one compares the penicillins containing large, sterically hindered, side chains, namely methicillin and oxacillin, one observes that methicillin retained the largest proportion of activity, whereas oxacillin, the better substrate for the wild-type β-lactamase, was more affected. The activity of both mutants drastically decreased (to $\leqslant 0.2\%$) towards all cephalosporins save nitrocefin. This sharp preference for nitrocefin when compared with other cephalosporins is similar to the behaviour of several natural class A β-lactamases, such as those of *Escherichia coli* (TEM1) and *Staphylococcus aureus*.

Most of our comparisons rest upon $k_{cat.}/K_m$ values which correspond to the second-order rate constant for acylation, k_{+2}/K', but when separate values could be obtained for $S^{130}A$, the $k_{cat.}$ values were more affected than were the K_m values. For $S^{130}G$, K_m values were always very high (except for cephalothin) and $k_{cat.}$ values were less affected. The observed specificity changes are difficult to interpret, because there were no evident correlations with the antibiotic structures. One should note, however, that the relationships between the amino acid substitutions and the specificities of the numerous class A β-lactamases remain completely mysterious at the present time. In the interaction with β-iodopenicillanate, particularly high K_m values were obtained (Table 4) for both mutants; indeed k_{+2} values were decreased to a lesser extent than K' values were increased, especially for $S^{130}G$. The postulated role of the invariant S^{130} in proposed catalytic mechanisms is not very clear. According to Moews *et al.* (1990), Ser^{130} would not make any direct contacts with the substrate, but would be important in positioning the β3 strand relative to the α2 helix, via an H-bond with Lys^{234}, thus playing a role in maintaining the geometry of the active site rather than a direct role in catalysis. This hypothesis totally disagrees with a suggestion of Oefner *et al.* (1990), who have recently reported the tertiary structure of a class C β-lactamase. Superimposition of that structure on that of a class A enzyme indicates that the hydroxy group of residue Tyr^{150} in the former lies in a position similar to that of the hydroxy group of Ser^{130} in the latter. The same authors hypothesize that this Tyr residue in its deprotonated form would act as does the His residue in the active serine proteinases of the chymotrypsin family, first accepting the Ser proton and then donating it back to the β-lactam nitrogen. During deacylation the Tyr anion would act as a general base, activating a water molecule for the nucleophilic attack on the acyl-enzyme. On the basis of the similar positions of the hydroxy groups of Tyr^{150} in class C and Ser^{130} in class A, the authors then suggest a possible equivalent role for Ser^{130} in class A enzymes, implying a dramatically decreased pK_a for the hydroxy group of this residue. Our results clearly show that Ser^{130} does not play such a role in class A enzymes, since the $S^{130}G$ and $S^{130}A$ enzymes retain a large proportion of their activity towards some substrates. If the first part of the hypothesis of Oefner *et al.*, (1990) is correct, one must assume that class C and class A β-lactamases, in spite of the good superimposition of many of their active-site residues, operate according to somewhat different catalytic mechanisms. In class A, Moews *et al.* (1990) and Herzberg & Moult (1987) have proposed a direct proton transfer from the active Ser^{70} to the β-lactam nitrogen facilitated by the proton repulsion due to the positively charged side chain of Lys^{73}. Alternatively, Glu^{166} might act as a general base catalyst (Ellerby *et al.*, 1990), but this would require a conformational change of the protein, bringing Glu^{166} close to Ser^{70} upon binding of the substrate. In both cases, the same residue would act as a general base in deacylation, by activating the incoming water molecule. Certainly, Glu^{166} in class A and Tyr^{150} in class C are not situated in similar positions relatively to the active-site elements which are common to both families of enzymes, i.e. Ser^{70}, Lys^{73} and the Lys^{234}-Thr^{235}-Gly^{236} triad in class A corresponding to Ser^{64}, Lys^{67} and the Lys^{315}-Thr^{316}-Gly^{317} triad in class C (Joris *et al.*, 1988). This would imply that two differently positioned residues would play comparable roles in the two families of enzymes. Indeed, with the presently available data, it seems safer to assume that Ser^{130} in class A maintains a functional active site by forming H-bonds with residues in the α/β domain. In that respect the importance of the side chain of residue 130 was also underlined by the generally poor recognition of β-iodopenicillanate and the increased K_m values for many substrates, which were particularly noteworthy with $S^{130}G$. Interestingly, in that latter case, the $k_{cat.}$ values for penicillins appeared to be less affected. Some of them were even close to those for the wild-type β-lactamase.

Position 131

The results of the mutations performed at this position demonstrated the role of Asp^{131} in protein stability. This amino acid is totally conserved among class A β-lactamases, although its side chain makes several contacts with non-invariant amino acids. In *S. albus* G β-lactamase this Asp is within H-bonding distance of several polypeptide backbone amido groups (132, 133, 134) and of other amino acid side chains (O. Dideberg, unpublished work).

The D-to-G mutation suppressed all these contacts and seemed to destabilize the protein severely, which would thus be unproperly folded and quickly proteolysed. Accordingly a very

low level of expression was observed. Such an underexpression has been described elsewhere for unstable mutant proteins (Schultz & Richards, 1986). Moreover, the time course of production was modified when compared with that of the wild-type and other mutant β-lactamases. The maximum of production appeared earlier, an observation which also correlated with a less stable mutant protein in other studies (Ellerby *et al.*, 1990).

The D-to-E mutation preserved all contacts, but the steric hindrance was increased. Since Asp^{131} is located in a loop rather than in a more stringent secondary-structure element, it could be expected that a structural adjustment might occur and allow the enzyme to maintain a good activity. This prediction appeared to be fulfilled (Table 5), and no large changes in specificity were observed; all substrates were hydrolysed, although somewhat less efficiently, and all K_m values increased, which probably reflected a slightly modified active-site cavity. Nevertheless, the protein stability was severely altered, irreversible denaturation occurring upon interaction of the protein with anion-exchangers. Thr^{71} is another active-site residue mainly involved in β-lactamase stability (Schultz & Richards, 1986). Asp^{131} seems to play an even more crucial role in stabilizing the protein structure, since the conservative D-to-E mutation strongly destabilized the protein. As expected from the fact that it points away from the substrate-binding site, residue Asp^{131} probably does not play a major role in substrate binding or catalysis.

Position 132

The dramatic effect of mutation $N^{132}A$ demonstrated the importance of Asn^{132} in catalysis. The need for an H-bond-forming side chain in that position was demonstrated by, first, the $k_{cat.}/K_m$ values for the best substrates of $N^{132}A$, which did not amount to more than 0.5% of those for the wild-type and, secondly, by comparisons of the activities of the $N^{132}A$ and $N^{132}S$ mutant β-lactamases, the latter being about 100-fold as active as the former. No major difference was observed in protein stability. Herzberg & Moult (1987) and Moews *et al.* (1990) postulated that Asn^{132} might act as an H-bond donor to the carbonyl group of the antibiotic side chain. If that H-bond were to be effective for ground-state binding, its disappearance upon replacement of Asn by Ala should result in a strongly increased dissociation constant. However, the catalytic constants ($k_{cat.}$) were distinctly more affected than the Henri–Michaelis constants (K_m), which seemed to indicate that Asn^{132} would be involved in transition-state stabilization rather than in ground-state binding. On the basis of the behaviour of the $N^{132}S$ mutant β-lactamase, an entropic effect in acylation was proposed for Asn^{132}. The fair-to-good activity of the $N^{132}S$ enzyme towards penicillins required that the development of an alternative H-bond between the antibiotic and Ser^{132} would result in a productive geometry for the nucleophilic attack by the active serine hydroxy group. In contrast, the resulting geometry with cephalosporins would be far less efficient. The loss of all H-bonds between the residue-132 side chain and the antibiotic due to the Asn-to-Ala mutation led to a similar conclusion: hydrolysis of cephalosporins requires a more accurate geometry, supplied in part by the correct amino acid in position 132. The loss of Asn^{132} side chain also resulted in a far less efficient hydrolysis of penicillins, but the adjustment by the enzyme was again better than for cephalosporins. At this point we would, however, emphasize an observation which is often overlooked when discussing the role of N^{132}: 6-aminopenicillanate is often a good substrate for class A β-lactamases, and both $N^{132}S$ and $N^{132}A$ mutations decrease the activity towards 6-aminopenicillanate more strongly than towards other penicillins. With that substrate, the H-bond postulated by Herzberg & Moult (1987) cannot be formed, and this would suggest a different role for that side chain.

The penicillin-binding proteins possess an SXN motif believed to correspond to the SDN one in class A β-lactamases, although no tertiary structures are presently available for any penicillin-binding-protein (Spratt & Cromie, 1988). A mutant of *E. coli* PBP3 exhibiting a large increase in cephalosporin resistance (6-fold towards cephalexin; 20-fold towards cephalothin and cefuroxime), but no increase towards benzylpenicillin and azthreonam, was shown to contain a $N^{361}S$ mutation (corresponding to N^{132}ABL) (Hedge & Spratt, 1985). This Asn^{361} might play a role similar to the Asn^{132} in class A β-lactamases and be involved in a good positioning of cephalosporins for efficient acylation.

Conclusion

All three positions of the 'SDN' loop of class A β-lactamases were modified by site-directed mutagenesis in order to understand the quasi-invariance of these three amino acids. All three are indeed important, either for the β-lactamase structure or good functioning, and their respective roles are quite distinct. Ser^{130} seems to help maintain the structure of the active-site cavity, Asp^{131} seems to be a key residue in protein structural stability, and Asn^{132} seems to act in the catalytic process. An interesting observation was that the mutations at positions 130 and 132 always decreased the cephalosporinase much more drastically than the penicillinase activity, with the exception of nitrocefin for the mutations at position 130. This might be correlated with the fact that class A β-lactamases are generally better penicillinases and might indicate that the hydrolysis of cephalosporin substrates require a more accurate complementarity between the active site and the substrate.

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