

Engineering a novel β -lactamase by a single point mutation

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β -Lactamases are widespread and efficient bacterial enzymes which play a major role in bacterial resistance to penicillins and cephalosporins. In order to elucidate the role of the residues lying in a conserved loop of the enzymatic cavity of the active-site serine *Streptomyces albus* G β -lactamase, modified proteins were produced by oligo-directed mutagenesis. Mutation of Asn116, which lies on one side of the active site cavity pointing to the substrate-binding site, into a serine residue resulted in spectacular modifications of the specificity profile of the enzyme. That replacement yielded an enzyme with a nearly unchanged activity towards good penicillin substrates. In sharp contrast its efficiency in hydrolysing cephalosporins was drastically reduced, the best substrates suffering the largest decrease in the second-order rate constant for serine acylation. In fact that single mutation generated a truly new enzyme behaving exclusively as a penicillinase, a situation which is never encountered to the same degree in any of the numerous naturally occurring variants of class A β -lactamases.

Key words: active site/ β -lactamase/site-directed mutagenesis/specificity/substrate profile

Introduction

Selective pressure due to a sometimes abusive utilization of penicillins and cephalosporins has been responsible for the emergence of an increasing variety of β -lactam hydrolysing enzymes (Bush, 1989). The molecular classification of β -lactamases proposed by Ambler (1980) rests upon their catalytic properties and primary structures. Class B enzymes are metallo-proteins which require a divalent cation (usually Zn^{2+}) for activity. However active-site serine β -lactamases, among which classes A, C and D are distinguished on the basis of their primary structures, represent the vast majority of β -lactamases. Their catalytic mechanism involves a nucleophilic attack of the β -lactam carbonyl by the serine hydroxyl, leading to the transient formation of an acylenzyme (scheme 1) (Fisher *et al.*, 1980; Knott-Hunziker *et al.*, 1982).

If the alignment of the known class A β -lactamases sequences (~15) indicates a very limited number of totally conserved residues (10% at most), very similar tertiary structures have been found for several enzymes (Kelly *et al.*, 1986; Dideberg *et al.*, 1987; Herzberg and Moulton, 1987; Samraoui *et al.*, 1986; Moews *et al.*, 1990), where many of the invariant residues are located around the active site cavity (Figure 1). Up to now the only attempts to position substrate models in the native enzyme active site have been performed by computer modelling and no direct observations of enzyme–substrate or enzyme–inhibitor

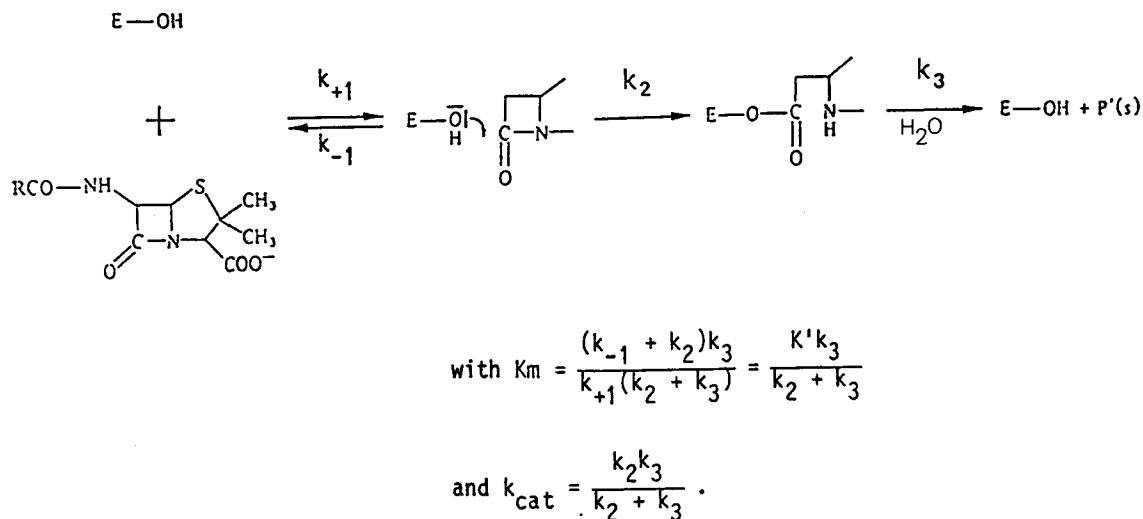
complexes have been reported. Consequently, although remarkable progress has been made recently in the knowledge of the molecular properties of β -lactamases, the proposed roles for the various active site residues which might collaborate with Ser70 (ABL) remain hypothetical. In particular the current hypotheses are very far from explaining the very large variations in the values of the catalytic parameters from one enzyme to the other with the same substrate or from one substrate to the other with the same enzyme. From the various side-chains modifications which have been performed chemically or by site-directed mutagenesis, the following conclusions can be drawn: (i) replacing Ser70 (ABL) (Figure 1) by Cys decreases the activity and alters the specificity profile (Sigal *et al.*, 1984); (ii) the side-chains of residues Lys73 (ABL) and Glu166 (ABL) are important, the latter might act as a general base catalyst (Little *et al.*, 1986; Madgwick and Waley, 1987); (iii) Lys234 (ABL) is involved in both ground-state and transition-state binding (Ellerby *et al.*, 1990).

So far, characterizations of the kinetic properties of mutant proteins have not been performed with a representative and extended sample of substrates. In this paper we present this type of detailed analysis for a mutant of the *Streptomyces albus* G β -lactamase. The chromosomal gene encoding that enzyme has been cloned and sequenced (Dehottay *et al.*, 1986, 1987) and the three-dimensional structure of *S. albus* G β -lactamase has been solved to 2.4 Å resolution (Dideberg *et al.*, 1987; O. Dideberg, unpublished data). The Ser-Asp-Asn motif (SDN loop, numbers 114–116 in *S. albus* G, ABL 130–132), situated between helices 4 and 5 (Figure 1) is nearly invariant in all class A β -lactamases. The crystallographic data indicate that the side chains of Ser114 and Asn116 point into the active site cleft, whereas that of Asp115 lies in the opposite direction, buried in a cluster of amino acids and involved in several H-bonds, probably fulfilling some structural role. We replaced all three residues in this conserved box by site-directed mutagenesis. Modifications of S114 or D115 proved to be detrimental to the β -lactamase, with respect to both activity and stability. Characterization of these mutants will be described elsewhere. The substitution of N116 by a serine yielded spectacular results.

Materials and methods

Chemicals

Enzymes for genetic engineering were purchased from Biolabs (Beverly, USA) and Boehringer (Mannheim, FRG); [³⁵S]dATP (1350 Ci/mmol) was from NEN (Boston, USA); crude ovalbumin was a gift from Belovo (Bastogne, Belgium). Benzylpenicillin was from Rhône-Poulenc (Paris, France), ampicillin and oxacillin from Bristol Benelux (Brussels, Belgium), 6-aminopenicillanic acid and carbenicillin from Beecham Research Laboratories (Brentford, Middlesex, UK), cefotaxime from Hoechst-Roussel (Romainville, France), cefazolin, cephalixin, cephaloglycin, cephaloridin, cephalosporin C and cephalothin from Eli Lilly and Co. (Indianapolis, IN, USA), cefuroxime from Glaxo Group Research (Greenford, Middlesex, UK). All antibiotics were



Scheme 1. Hydrolysis of a penicillin by an active site serine β -lactamase (E-OH).

kindly provided by the respective companies. Penicillanic acid was a gift from Pr Claes and Vanderhaeghe (K.U.L., Leuven, Belgium). Nitrocefin was purchased from Oxoid (Basingstoke, Hampshire, UK) and 7-aminocephalosporanic acid from Janssen Pharmaceutica (Beerse, Belgium). β -Iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, UK). FPLC columns for anion exchange and chromatofocussing were purchased from Pharmacia (Uppsala, Sweden).

Strains, plasmids and growth conditions

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 Altenb uchner (Regensburg, FRG) 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.

For the expression of the mutant protein, transformant *Streptomyces* colonies selected on thiostrepton R₂YE agar (Hopwood *et al.*, 1985) were used to inoculate 6-day cultures in modified YEME medium (Ercicum, 1990). Thiostrepton (25 mg/l) was added and baffled erlenmeyers were used. The pH was adjusted daily to 7.2.

Oligonucleotides

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

General methods

Recombinant DNA techniques were based on Hopwood *et al.* (1985) and Maniatis *et al.* (1982). DNA sequencing was carried out by the dideoxy method using the USB Sequenase Kit.

Site-directed mutagenesis

The following oligo was used to introduce the mutation: CTCCGACAGCTGCGCCG. Site-directed mutagenesis was performed using the 'Oligonucleotide-directed in vitro mutagenesis' kit from Amersham. After mutagenesis single-stranded DNA was prepared from randomly picked transformants and mutant clones identified by sequencing, using a primer which hybridizes with a stretch of DNA located ~150 bases upstream

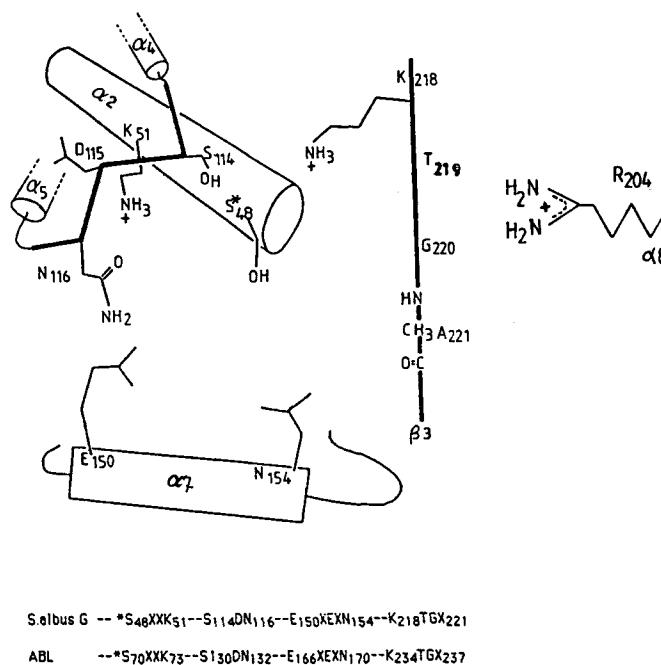


Fig. 1. (Top) Schematic representation of the active-site cavity of *S. albus* G β -lactamase. α refers to helices and β to a strand of a β -pleated sheet. (Bottom) Numbering of the active-site residues and corresponding numbers in the standard Ambler's classification (ABL). The one-letter code was used. * indicates the active serine and \times indicates a residue which is not conserved in all class A β -lactamases. When discussing general data about class A β -lactamases, we use Ambler's consensus numbering (ABL). When using specific numbering of the *Streptomyces* β -lactamase, we also give the corresponding number for the residue under investigation.

to the mutated site. Mutant clones were sequenced entirely to confirm that no undesired mutation had been introduced. This sequencing was performed using the universal M13 sequencing primer and four internal oligonucleotides.

Mutant β -lactamase purification

The mutant enzyme was purified from 3 l of culture supernatant as described for the wild-type protein (Matagne *et al.*, 1990) but the filtration on Sephadex G100 was omitted and a chromatography on a DEAE-Sephacel column was performed

after the chromatography on Q Sepharose. After DEAE-Sephacel an almost homogeneous preparation was obtained. Chromatofocussing as described for the wild-type enzyme (Matagne *et al.*, 1990) was performed to reach a high purity level. Enzyme homogeneity was demonstrated by the presence of a single band on SDS-PAGE. The enzyme was stored at -20°C in 50 mM sodium phosphate buffer, pH 7.0, containing 5% of each glycerol and ethylene glycol (EG/G 5/5). Protein concentrations were routinely determined by absorbance measurements at 280 nm. An extinction coefficient of 33 000/M.cm has been obtained with the wild-type enzyme.

Determination of the kinetic parameters of the mutant enzymes

When possible a complete time-course of the hydrolysis of the antibiotic was recorded at 482 nm for nitrocefin, 260 nm for other cephalosporins, and 235 nm for penicillins except for oxacillin (260 nm) and imipenem (300 nm). The values of the kinetic parameters K_m and k_{cat} were computed as described in De Meester *et al.* (1987). When the hydrolysis was too slow to obtain a complete time course within 5–10 min, initial 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_s in competition experiments using nitrocefin as a reporter substrate and initial rates measurements. 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). 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 an Uvikon 860 coupled to micro-computers via RS232 interfaces.

Results

Mutagenesis

The expression vector was prepared by cloning the fragment containing the entire *S. albus* G β -lactamase gene obtained by digestion of plasmid pDML6 (Dehottay *et al.*, 1986) by *Sst*I and *Pst*I into the modified pIJ702 (devoid of its unique *Kpn*I and *Bam*HI sites). The resulting plasmid was called pDML262. The *Kpn*I–*Pst*I 1.1 kb fragment, containing the 270 C-terminal amino acid residues of the β -lactamase coding sequence, was cloned into M13tg131 for mutagenesis.

Expression in *Streptomyces* and purification

The mutagenized insert was released from the M13 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 desired mutation in the final plasmid was confirmed by cloning the entire gene in M13mp19 and resequencing the region of the expected mutation. The derivative of pDML262 containing the mutation was used to transform *S. lividans* TK24, a strain devoid of intrinsic β -lactamase. The enzyme was purified from culture supernatant as described in Materials and methods. The behaviour of the mutant enzyme was similar to that of the wild-type one in all chromatographic steps although the yields during purification were repeatedly lower. The protein thus obtained was >90% pure as estimated by SDS-PAGE. No differences were observed in the electrophoretic mobility of the mutant when compared with the wild-type enzyme. Isoelectrofocussing revealed several active bands in similar positions for the wild-type and mutant enzymes.

Mutant β -lactamase stability

The thermal stability of the mutant protein 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 N116S mutant was quite stable although less so than the wild-type enzyme. The times for half-inactivation at 45°C for the wild-type and the N116S enzymes were respectively 55 and 20 min.

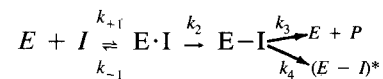
Kinetic parameters for several substrates

A detailed analysis of the kinetic properties of the mutant protein was then undertaken with a representative and extended sample of substrates, a type of study which has so far never been performed with mutant β -lactamases, and it will be seen that such an approach was extremely useful. The catalytic properties of the mutant enzyme were tested against a series of β -lactam antibiotics (Figure 2), chosen among the substrates studied with the wild-type β -lactamase (Matagne *et al.*, 1990). The penicillins formed a quasi continuous series in which the side chain was varied from penicillanic acid and 6-aminopenicillanic acid (or 6-APA) to the classical penicillins (benzylpenicillin, ampicillin, carbenicillin) and eventually oxacillin which contains a bulky side chain. A carbapenem (imipenem) and a compound exhibiting an imino side chain (mecillinam) were also included.

The cephalosporins contained various side chains on C3 and C7 (Figure 2). These compounds offered a wide range of variation in their qualities as substrates of the wild-type enzyme. Table I compares the kinetic parameters of the mutant to those of the wild-type enzyme. The introduced mutation was either neutral or detrimental to the enzyme. The modified enzyme lost >99% of its cephalosporinase activity but retained a good hydrolysing activity towards most penicillins.

Interaction with β -iodopenicillanate

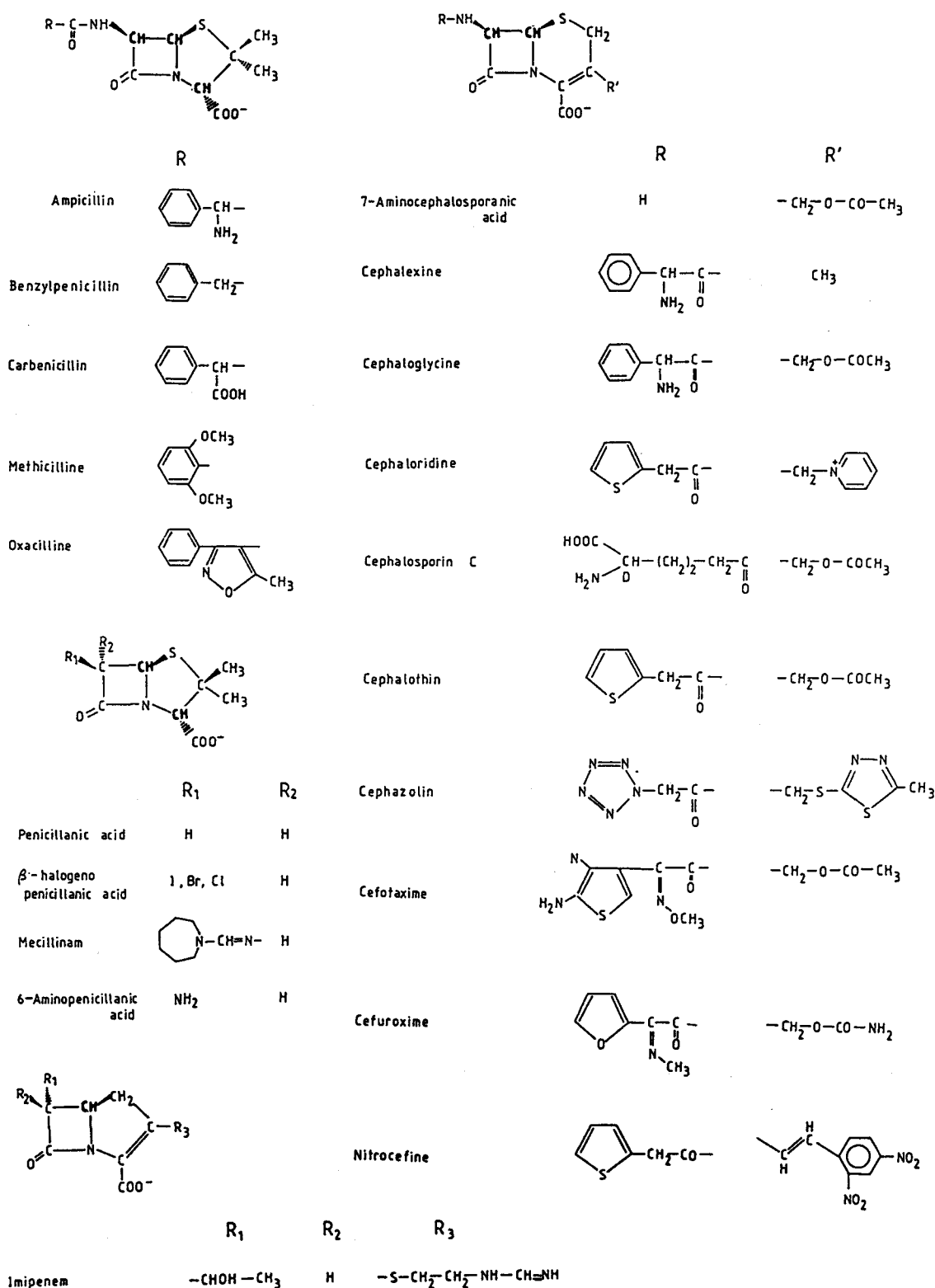
The suicide substrate (mechanism based inactivator) β -iodopenicillanic acid (β IP), which behaved as a real substrate for the wild-type β -lactamase was also tested against the mutant. For the wild-type enzyme, the interaction is known to occur according to the following pathway (Frère *et al.*, 1982):



The second-order rate constant for the formation of the acylenzyme (k_2/K') was 180 000 M.s for the wild-type enzyme, and it dropped to 500 M.s for the mutant β -lactamase. The k_3/k_4 ratio, which represents the ratio between the rate of hydrolysis of the acylenzyme and the rate of its rearrangement into an irreversibly inactivated complex, amounted to 515 for the wild-type enzyme and 12 for the N116S mutant. One can tentatively conclude that the acylation (k_2) is strongly impaired, and deacylation (k_3) is also reduced, so that the chemical rearrangement (k_4) of the acylenzyme occurs with a higher yield.

Docking of benzylpenicillin and cephaloridin in the active site of the enzyme

A penicillin (benzylpenicillin) and a cephalosporin (cephaloridin) both good substrates of the β -lactamase, were docked in the active site cavities of the wild-type enzyme structure and in a model structure of the N116S mutant. The distance between the hydroxyl of the active Ser48 (ABL70) and the β -lactam carbonyl group was kept constant, and the antibiotics were rotated to allow for the formation of an H-bond between the carbonyl of the antibiotic side chain and the side chain of residue 116 (NH for N116 or

Fig. 2. β -lactam antibiotics used in this study.

OH for S116). For the wild-type enzyme, the nitrogen atom of benzylpenicillin side chain formed an H-bond with the carbonyl group of residue 221 (ABL237) as described by Herzberg and Moul (1987) (Figure 3A). For the mutant, the shorter serine side chain prevented that latter interaction. By a slight rotation of the antibiotic, an alternative mode of binding appeared, with an H-bond between the nitrogen atom of the amide bond on the

antibiotic side chain and the carbonyl of Asn154 (ABL170) side chain (Figure 3B), necessarily more mobile than a portion of β -strand. The distances involved in those interactions are shown in Figure 4.

The same docking procedure was applied to the cephaloridin molecule but, since the orientation of the carboxylate group relative to the β -lactam plane is different in penicillins and

Table I. K_m , k_{cat} and k_{cat}/K_m values for several interactions

	WT enzyme ^a			N116S mutant			% WT
	k_{cat} (s)	K_m (mM)	k_{cat}/K_m (M.s)	k_{cat} (s)	K_m (mM)	k_{cat}/K_m (M.s)	
Penicillanic acid			61 000			1200	2
6-Aminopenicillanic acid	720	0.2	3.7×10^6	140	1.4	100 000	2.7
Benzylpenicillin	2800	1	2.8×10^6	1300	0.9	1.5×10^6	53
Ampicillin	3400	0.65	6.1×10^6	500	0.5	10^6	16
Carbenicillin			100 000			30 000	30
Oxacillin	270	0.43	630 000	>40	>5 ^d	8000	1.3
Imipenem	0.04 ^b	0.12 ^b	330 ^b	0.04	0.17	300	90
Mecillinam			2.6×10^{6c}			2.5×10^6	100
7-Aminocephalosporanic acid			170			5	3
Cephalexin	12	7.3	1700	$\cong 0.3$	$\cong 7^d$	40	2.3
Cephaloglycin	>100	>3	32 000	1.4	7 ^d	200	0.6
Nitrocefin			2.4×10^6			1200	0.05
Cephaloridine	260	0.32	620 000	0.6	1	600	0.1
Cephalothin	170	0.72	370 000	0.34	1.9	200	0.05
Cephalosporin C			40 000			130	0.3
Cefazolin	170	4.5 ^d	560 000	>2	>5 ^d	400	0.07
Cefuroxime			25 000			100	0.4
Cefotaxime			1000			<5	<0.5

^aValues obtained from Matagne *et al.* (1990). Standard deviations are calculated on 5–10 measurements and are usually within 10–20% of the value.

^bA. Matagne, unpublished.

^cDetermined in the present study.

^dDetermined by competition with a reporter substrate.

cephalosporins, a few short contacts were observed in the wild-type enzyme between substituents of the dihydrothiazine ring and the β 3-strand: for instance the distance between a carboxylate oxygen and the carbon of Gly220 (ABL236) was only ~ 1 Å. This indicates that the binding of cephalosporins probably requires a slight adjustment of the active site geometry. With the N116S mutant, these contacts were even shorter and prevented any binding without large changes in the protein structure. These results correlated well with the large decrease in cephalosporinase activity of our mutant.

Discussion

No gross perturbations of the enzyme structure by the chosen mutation were expected. That prediction was fulfilled, since the circular dichroism spectra of both the wild-type and the mutant β -lactamases were similar (not shown) as well as their isoelectrofocussing patterns; the mutant protein remained nearly as stable as the wild-type enzyme and retained a large proportion of its activity towards some substrates. Thus the asparagine residue does not appear to fulfil any essential function in the stability of the protein but plays a major role in the catalytic process, at least with substrates of the cephalosporin family. These conclusions were corroborated by the behaviour of a N116A mutant (not described here), that exhibited a stability similar to that of the wild-type protein but a drastically decreased activity towards all substrates, especially cephalosporins (its activity was 0.03–0.3% that of the wild-type towards penicillins, and nearly negligible (<0.001%) towards cephalosporins). Assuming that the same step remains rate-limiting for the wild-type and mutant enzymes, this amounts to a 4.1 kcal/mol increase in the free energy for that particular step with benzylpenicillin, underlining the importance of a potential H-bond donor in that position.

The mutant protein N116S was highly interesting since the dramatic modification of the specificity was due to the fact that the activity was either barely modified or largely decreased

depending upon the substrate under study. Indeed the k_{cat}/K_m values ranged respectively from 2 to 100% for penicillins, and from 0.05 to 3% of those of the wild-type for cephalosporins. In fact a truly new enzyme was generated by this single mutation, which exhibited a more pronounced specificity towards penicillins versus cephalosporins and a much more narrow spectrum of activity than any natural β -lactamase. Strikingly the decreases for the two molecules lacking an acyl side chain on the exocyclic nitrogen, 6-APA and 7-ACA (Figure 2) were very similar (~ 30 -fold), but penicillins were generally less affected than 6-APA, while all cephalosporins were more affected than 7-ACA. The mutant enzyme thus essentially became a penicillinase, the k_{cat}/K_m values for the best cephalosporin substrates (e.g. nitrocefin) being decreased to $\leq 0.1\%$ of those of the good penicillin substrates (benzylpenicillin, ampicillin and mecillinam).

In the cephalosporin series the decrease appeared to be remarkably dependent on the 'quality' of the substrate for the wild-type enzyme: the better the substrate the larger the decrease, so that the range of variation of the k_{cat}/K_m values was much more narrow for the mutant (5–1200/M.s) than for the wild type (170–2 400 000/M.s). When individual values of k_{cat} and K_M were obtained, it appeared clearly that the decrease of k_{cat}/K_m value was mainly due to a lowered k_{cat} value.

Herzberg and Moulton (1987) and Moews *et al.* (1990) have hypothesized that residue N132 (ABL) (N116 for *S. albus* G) might act as an H-bond donor to the carbonyl group of the antibiotic side chain, but the exact role of such a hydrogen bond remains obscure. When the potential H-bond former was modified (N116S) or eliminated (N116A) the kinetic parameter which was usually most severely affected was k_{cat} , which would rather indicate an effect on the stabilization of the transition state rather than on the binding of the ground state. Since that hypothetical H-bond would be rather a long way from the scissile C–N bond of the substrate, one might assume that it contributes to the adequate positioning of the substrate for the nucleophilic attack by the active serine O γ , suggesting mainly a strong entropic

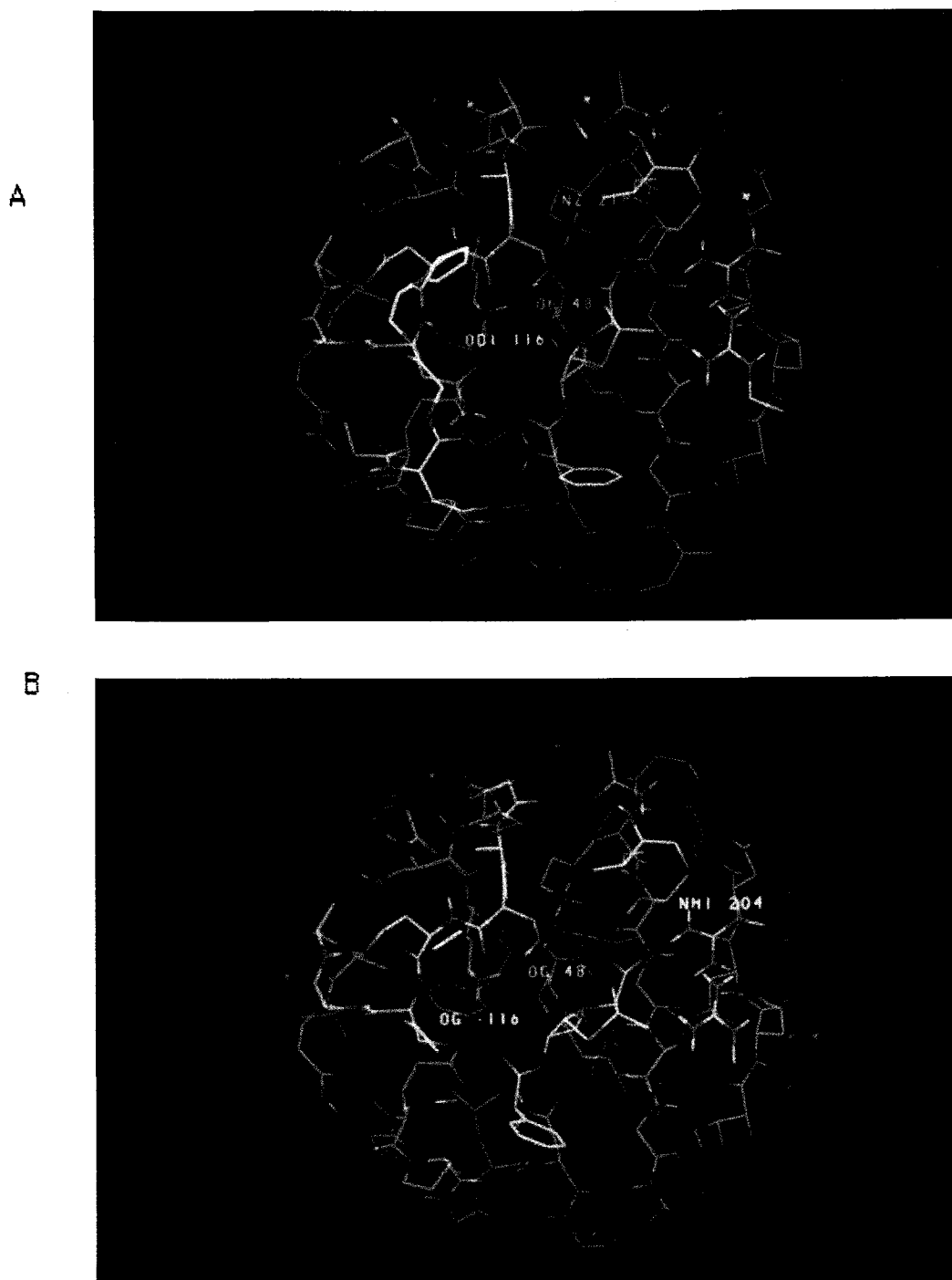


Fig. 3. Computer model showing the docking of benzylpenicillin in the active-site of the wild-type (A) and the N116S (B) enzymes. Colour code: yellow, carbon; red, oxygen; blue, nitrogen; green, sulphur. For the docking, the structure of benzylpenicillin and cephaloridin (not shown) were retrieved from the Cambridge data base (Allen *et al.*, 1979). Both molecules were docked using the graphic program FRODO (Jones, 1978) in the active site cavity of the enzyme structure refined at 1.7 Å resolution and in a model structure of the N116S mutant. All water molecules were removed and the side chains were kept in the same conformation except for Asn116 which was changed into a Ser in the mutant. After introducing the mutation, the torsional angle between C α and C β of the 116 side chain was set at the value observed for the wild-type protein.

effect. As shown by the docking attempts, formation of an alternative H-bond between benzylpenicillin and the new serine residue might still result in a productive geometry, while the geometric factors governing the attack of cephalosporins appear to be much more stringent. Indeed for cephalosporins which behave as poor substrates of the wild-type enzyme, the geometry is already not very productive with the asparagine residue and its replacement by the serine side chain is not too detrimental.

The same reasoning might also apply to imipenem, a very poor substrate also devoid of an acyl side chain.

With other penicillins the situation was more confused since the kinetic parameters were not similarly affected by the mutation. If the hypothetical hydrogen bond is important, one must first wonder why mecillinam and 6-APA, both unable to form such an H-bond, are such good substrates for the wild-type enzyme. Moreover, the mutation had a definite effect on the latter and

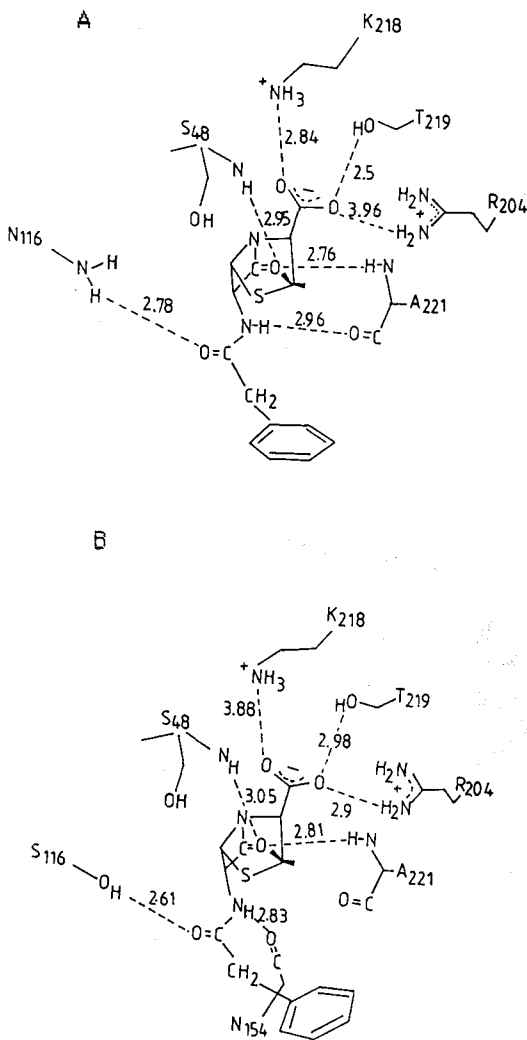


Fig. 4. Schematic representation of benzylpenicillin docked in the active site of the wild-type (A) and the mutant (B) β -lactamases. The distances (in Å) do not take account of H atoms.

none on the former. As for the cephalosporins, the modified parameter in the case of benzylpenicillin and ampicillin was k_{cat} . This was not true, however, for 6-APA (devoid of side chain) and oxacillin (with a bulky side chain), where a distinct increase in K_m was also observed, an observation as yet unexplained, unless the interaction between the wild-type enzyme and those two substrates was characterized by a limiting k_3 value (see below).

It is easier to explain on a purely kinetic basis how k_{cat} can be severely affected while K_m is only moderately modified on the basis of the generally accepted mechanism in scheme 1. A first possibility is that the mutations do not alter K' but decrease k_2 and k_3 by similar factors. However, a modification of k_2 alone might be sufficient to explain most of the results described here. Indeed in the analysis of the interaction between various class A enzymes and good penicillin substrates, Christensen *et al.* (1990) have shown that acylation (k_2) and deacylation (k_3) occurred at similar rates. This is probably the case of the *S. albus* G β -lactamase, for which the K_m values are usually very high, higher than those observed for the enzymes studied by Christensen *et al.* (1990), possible exceptions being 6-APA and oxacillin. Under those conditions a decrease of k_2 would result in a nearly proportional decrease of k_{cat} and k_{cat}/K_m , and at worst

a slight increase of K_m . This would agree with the proposed entropic effect of the N116 residue mainly affecting the acylation step. That the k_2 value might be more decreased by the mutations is also suggested by the results with β -iodopenicillanate. For the mutant N116S, the k_3/k_4 ratio suffered a significantly smaller decrease than the k_2/K' value.

The docking attempts also indicated for the wild-type β -lactamase the possible formation of an H-bond between the nitrogen atom of the amide bond on benzylpenicillin side chain (Figure 3) and the backbone carbonyl group of residue ABL237 (221 in *S. albus* G), which immediately follows the conserved KTG triad on the outermost strand of the β -pleated sheet (β_3 in Figure 1). This is in agreement with the suggestion of Herzberg and Moulton (1987). Similarly, Lys218 (ABL234), postulated by Herzberg and Moulton to be the electrostatic anchor of the antibiotic carboxylate group was 2.8 Å away from that group in the wild-type structure (Figure 4A). But, in the mutant structure, the distance between those two groups was too large (Figure 4B). This latter interaction could be replaced by a salt bridge between the carboxylate group and Arg204 (ABL220). This residue is not present in most class A β -lactamases, but as shown by the structure of the *Bacillus licheniformis* enzyme (Moews *et al.*, 1990) the side chain of Arg244, which is conserved in many β -lactamases (but not in the *S. albus* G enzyme) is situated in a similar position. It is interesting to note that Arg220 is often present when Arg244 is absent (B. Joris, unpublished). Moreover, mutations of Lys234 appear to lower k_{cat} rather than to increase K_m (Ellerby *et al.*, 1990; J. Brannigan and B. Joris, unpublished), indicating a role for Lys234 in the catalytic process rather than in the initial recognition. Since an alternative cationic centre can be supplied by one of the arginines described above, the usually accepted ideas about the ion pair partner of the substrate carboxylate should probably be revised.

The docking attempts also indicated that cephalosporins could not be introduced into the active site without minor adjustments of the wild-type protein structure. With the mutant, very large structural modifications became necessary, explaining an important loss of activity towards those substrates.

Among all the known class A primary structures, only that of β -lactamase III of *Bacillus cereus* presents a SDS sequence at position 130–132 (ABL) (Hussain *et al.*, 1987). That enzyme does not exhibit as strong a preference for penicillins as does our mutant (Connolly and Waley, 1983). Although residue 132 (ABL) plays an important role in determining the specificity profile of the enzyme, that profile is also strongly influenced by other side chains in the active site. Various modifications of the specificity have been reported by Hall and Knowles (1976), Sigal *et al.* (1984) and Sougakoff *et al.* (1989), due to mutations mainly in positions ABL237 and 238, just after the invariant KTG motif which forms one side of the active site pocket, while the SDN motif lies on the opposite wall. Recent data obtained with a natural variant of the RTEM-2 β -lactamase (Collatz *et al.*, 1989) indicated an increased activity towards ceftazidime and azthreonam when residue Arg164 (ABL) was replaced by serine (RTEM-7). Unfortunately, detailed kinetic studies are not available for any of those mutant or variant β -lactamases. Nevertheless the dramatic and coherent decrease of cephalosporinase activity was unique to our mutant.

Those results underline the interest of β -lactamases for site-directed mutagenesis studies: the large number of different parent enzymes and of available substrates can supply valuable information about the importance of minor modifications of the structures, various residues complementing or compensating for the influence

of others. Our studies also showed that it was necessary to determine the properties of mutants on a large range of substrates.

At the present time, we are trying to devise methods allowing the measurement of the individual rate constants k_2 and k_3 which will represent an additional step in understanding the mode of action of wild-type and mutant proteins.

This discussion might be relevant for other classes of β -lactamases and even penicillin-binding proteins and enzymes. The sequence alignments of Joris *et al.* (1988) indicate, in a situation similar to that of the 'SDN' loop of class A β -lactamases, the presence of a YSN sequence in the parent enzyme D-Ala D-Ala peptidase of *Streptomyces* R61 and a YAN sequence in class C β -lactamases. In penicillin-binding proteins, a careful examination of the sequences reveals a SXN sequence as a possible candidate (Spratt and Cromie, 1988). Thus in all cases the first and third residues of the triad are respectively a potential H-bond former (Ser or Tyr) and an asparagine. In the amino acid sequences the triad is always situated between the active serine and a negatively charged residue (Glu166 in class A β -lactamases) which in the known three-dimensional structures lies at the bottom of the active site and might serve as a general base in the catalytic process. It would thus be extremely interesting to analyse the influence of the replacement of these conserved residues on the behaviour of the other classes of penicillin-recognizing enzymes.

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