STUDIES ON THE CHROMOSOMAL BETA-LACTAMASE OF *PSEUDOMONAS AERUGINOSA.*

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The chromosomal β-lactamase of *Pseudomonas aeruginosa* SAI^{const} (a derepressed laboratory strain) was isolated and purified. Two peaks of activity were observed on gel permeation chromatography (one major peak mol. wt. 45 kD and one minor peak of 54 kD). Preparations from 12 clinical derepressed strains showed identical results.

Chromosomal β-lactamase production in both normal and derepressed *P. aeruginosa* strains was induced both by iron restricted growth conditions and by penicillin G. The majority of the enzyme (80-90%) was found in the periplasm and cytoplasm but a significant amount (2-20%) was associated with the outer membrane (OM). The growth conditions did not affect the distribution of the enzyme between subcellular fractions although higher activity was found in the cells grown under iron limitation and/ or in the presence of β-lactams.

The penicillanate sulphone inhibitor, tazobactam, displayed irreversible kinetics whilst cloxacillin, cefotaxime, ampicillin and penicillin G were all competitive inhibitors of the enzyme. Similar results were obtained for the *Enterobacter cloacae* P99 β-lactamase, but tazobactam displayed a non-classical kinetic pattern for the *Staphylococcus aureus* PC1 β-lactamase.

The residues involved in β-lactam hydrolysis by the *P. aeruginosa* SAI^{const} enzyme were determined by affinity labelling with tazobactam. A tryptic digestion fragment of the inhibited enzyme contained the amino acids D, T, S, E, P, G, A, C, V, M, I, Y, F, H, K, R. This suggests the involvement of the conserved SVSK, DAE and KTG motifs found in all penicillin sensitive proteins. A model of the 3-D structure of the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase was constructed from the published amino acid sequence of *P. aeruginosa* chromosomal β-lactamase and the α-carbon coordinates of the *S. aureus* PC1 β-lactamase by homology modelling and energy minimisation. The crystal structure of tazobactam was determined and energy minimised. Computer graphics docking identified Ser 72 as a possible residue involved in a secondary attack on the C5 position of tazobactam after initial β-lactam hydrolysis by serine 70.

The enhanced activity of tazobactam over sulbactam might be explained by the triazole substituent which might participate in favourable hydrogen bonding between N3 and active site residues.

Keywords: *Pseudomonas aeruginosa* SAI^{const}, chromosomal β-lactamase, tazobactam, molecular modelling, growth conditions, β-lactam antibiotics, β-lactamase inhibitors.

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ABBREVIATIONS

Å	angstrom
A_{459}	absorbance at 549 nm
BSA	bovine serum albumin
CDM	chemically defined media
CDM+Fe	iron-replete CDM
CDM-Fe	iron depleted CDM
CDM+Fe+PenG	iron-replete CDM containing penicillin G 500 mgl^{-1}.
CDM-Fe+PenG	iron-depleted CDM containing penicillin G 500 mgl^{-1}.
CF	cystic fibrosis
cfe	cell free extract
cfu	colony forming unit
cm	centimetre
CM	cytoplasmic membrane
conc	concentrated
CPase	carboxypeptidase
℃	degree centigrade
°	degree
deg min^{-1}	degrees per minute
DNase	deoxyribonuclease
E	enzyme
$[E_0]$	initial enzyme concentration
E.S	Michaelis complex
E-S	acyl-enzyme
E-I	enzyme-inhibitor complex
E-I*	irreversibly inhibited enzyme (dead-end complex)
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
e.s.d.	estimated standard deviation
FPLC	fast protein liquid chromatography
g	gram
g	gravity
h	hour
Hom	homogenate
HPLC	high pressure liquid chromatography
I	inhibitor
$[I_0]$	initial concentration of inhibitor
[I]	inhibitor concentration
IEF	isoelectric focusing
IRMP	iron regulated membrane protein
kcal	kilocalories
k_{cat}	rate constant or turnover number
kD	kilodaltons
K_i	inhibitor constant
kJ	kilojoule
K_m	Michaelis constant
l	litre
M	molar
mA	milliamp
µg	microgram
mg	milligram
MIC	minimum inhibitory concentration

min	minute
µl	microlitre
ml	millilitre
mol. wt.	molecular weight
Mole.l^{-1}	moles per litre
MOPS	3-(N-morpholino) propane sulphonic acid
Mr	relative molecular mass
mm	millimetre
µm	micrometre
mM	millimole
µM	micromole
NB	nutrient broth
NB + Pen G	nutrient broth containing penicillin G 500 mgl^{-1}
NCTC	National Collection of Type Cultures
nm	nanometre
OD	optical density
OD_{420}	optical density at 420 nm
OD_{750}	optical density at 750 nm
OM	outer membrane
OMP	outer membrane protein
P	product
PAGE	polyacrylamide-gel electrophoresis
PBP	penicillin-binding protein
pCMB	p-chloromercuriobenzoate
PG	peptidoglycan
pI	isoelectric point
PIA	*Pseudomonas* isolation agar
Rf	retardation factor
RNase	ribonuclease
rpm	revolutions per minute
s	second
S	substrate
[S]	substrate concentration
SA	specific activity
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
spp	species
TA	total activity
TEMED	tetramethyl ethylamine solution
TM	total membranes
TMP	total membrane protein
TP	total protein
TPase	transpeptidase
TRIS	tris(hydroxymethyl) aminomethane
TSB	tryptone soya broth
V	velocity
V_{max}	maximum velocity
W	Watt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

1. INTRODUCTION

1.1 Background to the project and aims of the study

β-Lactams are the most commonly used group of antibiotics and it is this high frequency of usage that has promoted the development of β-lactamase resistance mechanisms in pathogenic bacteria. β-Lactam antibiotics penetrate the OM of Gram-negative bacteria via aqueous porin channels (Nikaido and Vaara, 1985; Nakae, 1986) and bind to specific PBP targets located in the CM of bacteria (Spratt, 1979; 1983) where they exert their inhibitory effects by distorting the cell shape and eventually causing lysis (Noguchi *et al.*, 1979a; Noguchi *et al.*, 1979b; Spratt, 1979). β-Lactamases are a ubiquitous group of bacterial enzymes and their capability for hydrolysing the β-lactam bond makes them a major cause of resistance to β-lactam antibiotics (Livermore, 1987).

Many distinct types of β-lactamase have been demonstrated (Medeiros, 1984) and numerous schemes proposed for classifying them (Medeiros, 1984; Richmond and Sykes 1973). The most useful technique currently available for the identification of β-lactamases is isoelectric focusing (Matthew and Harris, 1976; Matthew *et al.*, 1975, Vecoli *et al.*, 1983) in which the banding pattern produced acts as a unique fingerprint for each enzyme. This technique has identified over 50 different β-lactamases. Some are encoded by genes carried on plasmids or transposons whilst others are encoded by chromosomal genes (Datta and Kontomichalou, 1965; Hedges and Jacob, 1974). The variety of substrate specificities, the large number of encoding genes and gene mobility coupled with changing patterns of drug usage and advances in antibiotic design have lead to changes in the importance of each type of β-lactamase at different times.

During the 1940-1950s the available β-lactams were directed against Gram-positive species, which led to the emergence of penicillinase-producing staphylococci (Richmond, 1965). In the 1960s the development of various anti-Gram-negative broad spectrum penicillins and cephalosporins led to an increased prevalence of ampicillin-resistant bacilli such as *Escherichia coli*, *Salmonella*, *Klebsiella* and *Haemophilus*. This resistance was largely determined by plasmid-mediated β-lactamases. These compounds were susceptible to plasmid-mediated β-lactamases and particularly to the TEM-1 type enzyme. First recognised in a few isolates of *E. coli* this enzyme rapidly spread amongst enterobacteria and then spread to *P. aeruginosa*, *Haemophilus influenzae* and

Neisseria gonorrhoeae in by the 1970s reducing the usefulness of amino- and carboxy-penicillins against *enterobacter*, *H. influenzae* and *P. aeruginosa* and benzyl penicillin against *N. gonorhoeae*. Matthew *et al.* (Matthew *et al.*, 1975; Matthew, 1979a; 1979b; Matthew and Harris 1976) described 11 types of plasmid-mediated constitutively-produced β-lactamase which could be classified according to their substrate specificity: broad spectrum penicillinases; oxacillinases; and carbenicillinases. All enzymes gave high level resistance to ampicillin and carbenicillin as well as low level resistance to most of the early cephalosporins.

In 1983 *Klebsiella* strains were isolated in West Germany that produced a plasmid-mediated β-lactamase capable of hydrolysing cefotaxime and several of the newer cephalosporins (Kleibe *et al.*, 1985). The enzyme, SHV-2, had arisen by mutation from SHV-1 (a common enzyme in *Klebsiella* spp.). SHV-2 has now spread to countries as far apart as Chile, France and Greece (Medeiros and Hare 1986). More recently a new plasmid enzyme, CTX-1, derived from TEM-2, has been found which also confers resistance to cefotaxime (Brun-Buisson *et al.*, 1987; Sirot *et al.*, 1987; Goussard *et al.*, 1987). Hence, the selection pressure exerted by widespread use of third generation cephalosporins has led to the evolution of transferable β-lactamases with extended spectra of activity.

Following the increased use of third generation cephalosporins a new resistance problem is now emerging. High levels of resistance among species that produce inducible chromosomally-determined β-lactamases are appearing (Sanders, 1983; Vecoli *et al.*, 1983; Olson *et al.*, 1983). This phenomenon, which was seldom encountered prior to the introduction of third generation cephalosporins has emerged in clinical isolates during antibiotic therapy. Mutations in the genes regulating β-lactamase production occur with a frequency of 1 in 10^6 or 10^7 and lead to constitutive production of chromosomal β-lactamases. Such mutations appear most commonly in *P. aeruginosa* and *E. cloacae* and reports are being made of therapeutic failures and relapses of infection due to stable partially or fully derepressed β-lactamase producing strains of these organisms (reviewed by Sanders, 1987).

Resistance to the newer cephalosporins mediated by chromosomal β-lactamases appears to be both a function of the rate of hydrolysis of the antibiotic and of the poor

ability of the agent to permeate the OM of the bacterial cell (Sugarman and Pesanti, 1980; Vu and Nikaido, 1985). The result is that isolates are resistant to third generation antibiotics, but susceptible to penems and carbapenems such as imipenem (Sanders, 1983), which rapidly penetrates the OM and is resistant to hydrolysis (Nikaido, 1985).

1.2 Clinical importance of β-lactamase

The incidence of clinical problems occurring because of β-lactamases in certain bacteria is rising. Therapeutic failures have been linked to the development of multiple resistance in strains for the newer β-lactams (Sanders and Sanders, 1985; Collatz *et al.*, 1984; Weinstein, 1985; Mall *et al.*, 1985; Follath *et al.* 1987; Dworzack *et al.*, 1987). Organisms with multiple resistance for the newer β-lactams are spreading within the hospital environment and are becoming important nosocomial pathogens. The biggest problems are encountered where patients are highly susceptible to infections e.g. *P. aeruginosa* infections in CF centres and *P. aeruginosa, Enterobacter spp.* and *Serratia spp.* infections in intensive care and burns units. Patients failed to respond to therapy or relapsed despite the susceptibility of bacterial isolates recovered during the initial stages of therapy. Later cultures revealed that stably induced mutants resistant to multiple β-lactam antibiotics had been selected from the initially susceptible strain. Combination drug therapy of infections has not prevented the emergence of resistant strains (Sanders and Sanders, 1985; Follath *et al.*, 1987; Winston *et al.*, 1984).

Virtually all Gram-negative bacilli produce chromosomally-mediated β-lactamases (Matthew, 1979; Medeiros, 1984; Sykes and Matthew, 1976). The most important of these are the class C enzymes (Ambler, 1980) produced by *P. aeruginosa* and clinically-significant Enterobacteria excepting the Klebsiellae (Richmond and Sykes, 1973) and Salmonellae. The enzymes preferentially hydrolyse cephalosporins but can inactivate penicillins (Sawai *et al.*, 1982). Bacteria possessing β-lactamases may produce increased levels of enzyme in either of two ways:

(1) Induction by the presence of an inducing β-lactam (Livermore, 1987; Sanders and Sanders, 1986; Then, 1987). The organism produces elevated levels of β-lactamase only as long as the inducing β-lactam stays in the environment.

(2) Mutation to the stably derepressed state (Livermore, 1987; Sanders and Sanders, 1986; Cullmann *et al.*, 1987). The high levels of enzyme are produced regardless of the

presence or absence of an inducer.

The drugs that are resisted by these bacteria once induction of the β-lactamase occurs include most of the newer cephalosporins, cephamycins, monobactams and expanded-spectrum penicillins. The broad degree of cross-resistance mediated by these enzymes is surprising since many of the newer drugs resisted are considered to be β-lactamase stable. Studies have shown that multiple β-lactam resistance is due to the enzymes themselves (Gootz *et al.*, 1984; Gootz *et al.*, 1982; Lampe *et al.*, 1982; Livermore *et al.*, 1982; Sanders and Sanders, 1985; Livermore, 1987; Then and Angehrn, 1982; Vu and Nikaido, 1985; Werner *et al.*, 1985; Cullmann *et al.*, 1987; Gutmann and Williamson, 1983; Seeberg *et al.*, 1983; Phelps *et al.*, 1986). The presence of large numbers of β-lactamase molecules inside the periplasmic space following induction or mutation, combined with the high affinity of these enzymes for many β-lactams is sufficient to mediate resistance even to poor-substrate β-lactams.

This problem is potentially serious. There are now reports concerning treatment failures of infections caused by organisms with inducible β-lactamases treated with newer cephalosporins (Weinstein, 1985; Mall *et al.*, 1985; Dworzack *et al.*, 1987; Winston *et al.*, 1984; Nichols *et al.*, 1983). In these reports, resistance developed during or after therapy in approximately one third to one half of patients infected with *P. aeruginosa*, *Enterobacter* spp. or *Serratia marcescens*, all of which possess inducible β-lactamases (Sanders *et al.*, 1987).

Outbreaks have been reported that involved nosocomial infections due to multiply β-lactam resistant *P. aeruginosa* in CF centres during periods of heavy use of the newer β-lactam antibiotics e.g. Pedersen *et al.* reported an epidemic of multiply-resistant *P. aeruginosa* in their CF centre (Pedersen *et al.*, 1986). This epidemic was associated with the extensive use of third generation cephalosporins in the centre. The use of first and second generation β-lactams was recommended to avoid similar problems arising again.

1.3 Mechanism of action of β-lactams upon sensitive enzymes

The final stage of peptidoglycan synthesis involves the translocation of the disaccharide peptide unit across the CM and transfer to an non-cross-linked linear glycan strand (a transglycosylation) followed by cross-linking of the peptide chain to

the existing PG by transpeptidation. The transpeptidase reaction is the major site of β-lactam action. β-Lactams act as substrate analogues by binding to and acylating the L-serine residue in the active site of the transpeptidase enzymes and form stable complexes, thereby preventing further transpepide cross-linking and leading to cell lysis and death.

The molecular interaction between β-lactams and their targets has been elucidated using *Streptomyces* R61 and R39 carboxypeptidases as model penicillin-sensitive enzymes and has been found to be more complex than first thought. Cells have several types of PBP with different functions and sensitivities (Tipper, 1985). Knowledge of the sequence and tertiary structure of the active sites of the PBPs, from which β-lactamases are probably evolved, may help in the design of new β-lactamase inhibitors. Considerable active site sequence homology has been demonstrated between the β-lactamases and penicillin-sensitive CPases and TPases (See Table 1). β-Lactam antibiotics and acyl-D-Ala-D-Ala substrates acylate the same active site serine residue in a number of PBPs from different organisms (Georgopapadakou *et al.*, 1981; Waxman and Strominger, 1980; Yocum *et al.*, 1982; 1979).

Boyd (1982) has shown that, for a compound to be a good antibiotic, it must have a sufficiently reactive β-lactam bond to acylate PBP sites effectively. However, while necessary to ensure acylation, such reactivity is not sufficient to ensure potency. Potency will depend on the degree of "fit" to the active site and on how resistant the acyl-enzyme is to hydrolysis. The degree of "fit" in turn will depend on how well the antibiotic compound mimics the normal acyl-D-Ala-D-Ala substrate.

β-Lactam antibiotics are effective enzyme inhibitors (Ghuysen *et al.*, 1980) which are thought to participate in the following reactions

$$E + \text{penicillin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{penicillin-E} \xrightarrow{k_3} \text{penicilloyl-E} \xrightarrow{k_4} \text{E-active} + \text{penicilloic acid}$$

Good inhibitors were found to be the ones that trapped the enzyme (E) in an enzyme antibiotic complex quickly (i.e. with a high k_3 value) and for a long time (i.e.low k_4 value). Hence it is acylating efficiency (k_3) rather than binding affinity (k_1) that is paramount in PBP inhibition. Kinetic parameters alone cannot predict the nature or

antibacterial effect of a compound (e.g. bactericidal potency). A complicating factor is the continued production of new PBPs by penicillin treated cells (Tomasz, 1986).

β-Lactam antibiotics kill bacteria by inactivation of certain PBPs. The selective inhibition of PBPs has been most extensively studied in temperature-sensitive mutants in *E. coli* (Spratt, 1977b). However, many β-lactam antibiotics have similar affinities for several PBPs, and at concentrations above their MIC, may kill the cell by inactivating more than one type of PBP. To determine the mechanism of lethality for inhibition of individual species of PBP, the concentration of β-lactam antibiotic that saturates or has an affinity for only one type of PBP has to be investigated. Cephaloridine and cefsulodin saturate PBP1A and PBP1B before PBP2 or PBP3. These antibiotics cause rapid and extensive killing of *E.coli* with degradation of the cell wall material and rapid lysis. Mecillinam, clavulanic acid and thienamycin derivatives have a high affinity for PBP2, which is saturated at their MIC. These antibiotics convert *E. coli* to osmotically stable ovoid cells, followed by loss of viability, but not accompanied by extensive cell wall degradation. Cephalexin and aztreonam are highly selective for PBP3 and cause selective inhibition of septation. This results in formation of long filaments but only a slight loss of viability and lysis. (Spratt, 1977a). Low concentrations of many β-lactams; penicillin G, ampicillin, and cephalothin saturate PBP3 before any other PBPs. At these concentrations filamentation is also seen, however at higher concentrations above their MICs swelling and lysis of the cells occurs which corresponds to saturation of PBP1A at these concentrations. PBP3 does not appear to be a target for the action of β-lactams, if a β-lactam attacks only PBP3, then bacterial death does not inevitably follow. If *E. coli* is treated with cephalosporins at concentrations only inhibitory for PBP3 (Chase *et al.*, 1981) the cells stop dividing but continue to grow as non-septate filaments. Removal of the inhibitor results in resumption of cell division by break-up of the filaments into normal-sized viable cells. Lysis is only seen when the concentration of cephalosporin is increased so that it combines with the PBP1 complex (Chase *et al.*, 1981). The high *in vivo* efficacy of aztreonam (Bonner *et al.*, 1981) may be related to some aspect of the filamentous morphology that is disadvantageous for the bacterium *in vivo*, e.g. increased susceptibility to phagocytosis (Andreana *et al.*, 1984).

Table 1. Active site residues of penicillin recognising enzymes.

Class	Enzyme	Sequence
		*
	CPase B	FGVGSLSK
		FGVGSVTK
	CPase PBP5 *E.coli*	LGIASMTK
	CPase *B.stearothermophilus*	RDPASLTK
	CPase *B.subtilis*	LGIASMTK
	TPase PBP3 *E.coli*	FEPGSTVK
A	β-lactamase	
	S. aureus	FAYASTSK
	B.licheniformis	FAFASTIK
	B.cereus I	FAFASTYK
	Strep.albus G	FPMGSVPK
	pBR322 β-lactamase	FPMMSTFK
	K.aeruginosa KI	FAMNSTSK
C	β-lactamase	
	E. cloacae P99	FELGSISK
	P. aeruginosa	FELGSVSK
	E. coli chromosomal	FAYASTSK
	MnHI	FELGSISK
D	β-lactamase	
	carboxy-terminal of penicillin	
	receptor involved in β-lactamase	
	induction in *B. licheniformis*	FAPASTYK
	OXA 2	YSPASTFK
	Plasmid mediated OXA-1	--PDSTFK
	PSE 2	--PASTFK
	High Mr PBP 1A of *E. coli*	LRVGSNIK
	PBP 1B of *E. coli*	RRIGSLAK
	PBP 2 of *E. coli*	YPPASTVK
	PBP 3 of *E. coli*	FEPGSTVK
	Low Mr PBP	
	B. subtilis	LPIASMTK
		*

* Denotes active site serine

Active site sequences of penicillin recognising enzymes aligned by active site serine residue. All active sites contain the SxxK motif.

Autolysin activation in bacteria has been implicated in death due to exposure to β-lactam antibiotics where lysis occurs (e.g. in death caused by inactivation PBP1A and PBP1Bs in *E. coli*). The association of the bactericidal action of penicillin and activation of autolytic enzymes has been shown with *Strep. pneumoniae* (Horne and Tomasz, 1980; Tomasz, 1979a; 1979b; 1980; 1986; Williamson and Tomasz, 1980). Autolysin activation may involve a number of sequential steps, whose control is tied to the cell growth cycle (Williamson and Tomasz, 1980).

1.4 Evolution and function of β-lactamases

β-Lactamases may have evolved from PBPs involved in PG synthesis (Pollock, 1967; Tipper and Strominger, 1965). This theory is supported by the sequence homology between the class A enzymes and the D-alanine carboxypeptidases of *Bacillus* species. Sequence homology has also been demonstrated between these enzymes and PBP5 and PBP6 of *E. coli* (Waxman and Strominger, 1983). Kelly *et al.* (1985) have alsó shown similarities between the sequences immediately adjacent to the active site serines of the penicillin sensitive D-alanine carboxypeptidase-transpeptidase from *Streptomyces* R61 and class C β-lactamases. No sequence homology was detected between the class A and class C β-lactamases and so it has been concluded that they evolved independently (Kelly *et al.*, 1985).

β-Lactamases and PBPs also share functional characteristics. Recently it has been shown that like many β-lactamases, the low affinity PBP of methicillin-resistant *S. aureus* is inducible upon exposure to β-lactam antibiotics (Chambers *et al.*, 1985; Ubukata *et al.*, 1985). Although some PBPs have weak β-lactamase activity (Amaral *et al.*, 1986) the reverse has not yet been demonstrated, however, Abraham and Chain (1940) did propose that β-lactamases might have some function in the cell metabolism.

β-Lactamases are produced in bacterial strains isolated before β-lactam antibiotics were used in antibacterial chemotherapy (Pollock, 1967) and are present both in β-lactam susceptible and β-lactam resistant bacteria (Onishi *et al.*, 1974). Possibly, β-lactamases may fulfil some, as yet unknown function in bacterial cell metabolism. Whatever the function or origin of β-lactamases, it is clear that the clinical use of β-lactam antibiotics has played a major role in their widespread distribution and further evolution.

1.5 Classification of β-lactamases

In 1980 Ambler (1980) proposed the first classification scheme based on molecular structure. Examination of the four amino acid sequences available at that time revealed homology in key sequences among *S. aureus* PC1, *B. cereus* I and *B. licheniformis* penicillinases and the RTEM broad spectrum β-lactamase, but little homology between these enzymes and the zinc-containing *B. cereus* II cephalosporinase. He proposed two classes: A - the penicillinases or broad spectrum enzymes later shown to contain an active site serine; and class B - the metalloenzymes. Jaurin and Gundstrom (1981) expanded the scheme to include class C enzymes, which also contained an active site serine, had a distinct amino acid sequence around the active site (Fisher *et al.* 1980) but which were cephalosporinases. A final class was added by Huovinen *et al.* (1988) who sequenced the PSE-2 β-lactamase (Huovinen *et al.* 1988). Based on the similarities between PSE-2 and OXA-2 enzymes and their lack of similarity to the TEM or chromosomal lactamases (Jaurin and Grundstrom, 1981; Sutcliffe 1978), they were assigned a class of their own - class D.

Class A contains the β-lactamases from *S. aureus*, *B. licheniformis* , *B. cereus* (β-lactamase I) and β-lactamase from *E. coli* TEM. These enzymes showed extensive amino acid sequence homology, a reactive serine (later shown to be Ser 70 according to the Ambler (1980) numbering scheme) and conserved lysine residue (Lys 73), were all penicillinases (Collatz *et al.*, 1990), have a molecular weight of 29 kD and form an acyl-enzyme complex that results in hydrolysis of the β-lactam (Fisher *et al.*, 1980; Cartwright and Coulson, 1980; Yocum *et al.*, 1979; Kuwabara and Abraham, 1967; Davis and Abraham, 1974; Ambler, 1980). Class A enzymes show homology with the amino acid sequences of *B. stearothermophilus* and *B. subtilis* D-alanine carboxypeptidases (Bush and Sykes, 1984) which suggests an evolutionary link between these groups of enzymes.

Class B enzymes are metalloenzymes that show little or no homology with the class A β-lactamases and therefore have been assigned to a class of their own (Jaurin and Grundstrom, 1981). They are unique since they destroy compounds containing a 7-α-methoxy group, require a metal cofactor (Zn^{2+}) for activity (Bergstrom *et al.*, 1983), and are not susceptible to any inhibitors except chelating agents that bind zinc.

Examples include *B. cereus* II β-lactamase and the β-lactamase from *P. maltophilia* .

Class C enzymes showed no sequence homology with those of class A or B and so these were placed in a class of their own (Ambler, 1980). Most enterobacteria and related species express a class C chromosomally-encoded (*ampC*) β-lactamase (Neu, 1983). The complete amino acid sequence of *E.coli* K12 β-lactamase has been determined by sequencing its structural gene. This enzyme has a molecular weight of 39.6 kD and shows extensive sequence homology with *P. aeruginosa* chromosomal β-lactamase (Lodge *et al.*, 1990). The chromosomal β-lactamase gene of *Citrobacter freundii* has been cloned (Bartolene *et al.*, 1984) and sequenced (Schenkein and Pratt, 1980) and comparisons of this gene with those from *E. coli* K12, *P. aeruginosa* and *Shigella sonnei* (Borders *et al.*, 1982) reveals extensive sequence homology.

Class D β-lactamases contain the serine-threonine-phenylalanine-lysine (STFK) tetrad also found in the active site of the TEM-1 β-lactamase, but otherwise show very little sequence homology with this group of enzymes (Huovinen *et al.*, 1988). See Table 2.

Table 2. The Ambler classification scheme for β-lactamases

Class	Example
A	e.g. *S. aureus, B.licheniformis, B. cereus* I, *E. coli* TEM Key amino acid: serine e.g. *B. cereus* I
B	Metallo-enzyme, zinc, inactivate moxalactam, imipenem and cefoxitin
C	e.g. *P. aeruginosa* Chromosomally encoded ampC gene of *E. coli* K12 Key amino acid: serine
D	e.g. OXA plasmids Key amino acid: serine

No classification scheme is yet completely ideal. Complications have arisen with the demonstration of plasmid-encoded cephalosporinases which have the characteristics of chromosomal enzymes (Levesque *et al.* 1982, Sirot *et al.*, 1987), the discovery of

chromosomally-located transposons that encode β-lactamases (Sinclair and Holloway, 1982) and the finding of the supposedly *Pseudomonas* specific enzyme PSE-2 in several enterobacteria species (Livermore *et al.,* 1984). New enzymes are constantly being found. The advent of the second and third generation cephalosporins has produced new classification schemes and terminology which add to the confusion. The terminology of the Ambler classification scheme will be used in this work.

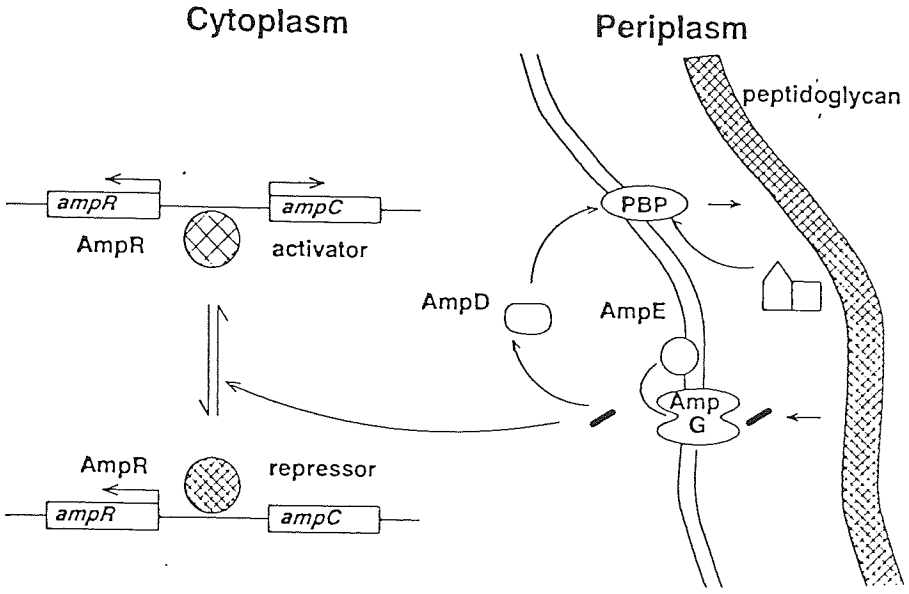
1.6 Molecular basis for the induction of β-lactamase

The most detailed information on the control of β-lactamase expression has been obtained from studies in *E. coli* and other enterobacteria. Since the nucleotide sequence of the *E. coli ampC* gene was determined (Jaurin and Grundstrom, 1981) it has become apparent that this chromosomal β-lactamase belongs to the class C or serine β-lactamases. Most enterobacteria and related organisms possess an *ampC* gene coding for chromosomal β-lactamase (Bergstrom *et al.,* 1982; Bergstrom *et al.,* 1983; Lindberg and Normark, 1986á; Galleni *et al.,* 1987; Burman *et al,.* 1973; Nomura and Yoshida, 1990). In species such as *C. freundii*, *E. cloacae*, *S. marcescens*, some indole positive *Proteae* and *P. aeruginosa*, production of the Amp C β-lactamase is induced by β-lactam compounds (inducible),whereas in others such as *E. coli* and *Shigella sonnei* it is not (β-lactamase is constitutively expressed) (Lindberg and Normark, 1986b; Richmond and Sykes, 1973; Sykes and Matthew 1976; Sanders, 1987). The basic difference between inducible and constitutive β-lactamase producers is the possession of a regulatory gene, *ampR*, which has been shown to be present in the inducible species in which a basal level of β-lactamase expression can be induced more than 100 fold by some β-lactams, e.g. *C. freundii*, but lacking in non-inducible organisms, where the presence of a β-lactam has no effect on expression, e.g. *E. coli* (Bergstrom *et al.,* 1983; Lindberg *et al.,* 1985; Curtis *et al.,* 1986).

The *ampR* gene codes for a 31 kD DNA-binding protein (AmpR) (Lindberg *et al.,* 1985; Honore *et al.,* 1986; Lindquist *et al.,* 1989) which binds upstream from the *ampC* promoter where it can block the transcription of the *ampC* gene. The promoter regions for *ampC* and *ampR* are situated close to one another in *E. cloacae* (Honore *et al.,* 1986). As well as controlling transcription of the *ampC* gene, AmpR controls transcription of the *ampR* gene (Lindberg *et al.,* 1985; Lindquist *et al.,* 1989; Nicolas

et al., 1987). See Figure 1.

Figure 1. Induction of ampC β-lactamase in *Enterobacter*



In the non-induced state AmpR binds upstream from the *ampC* promoter where it blocks the transcription of the *ampC* gene, basal levels of β-lactamase may be produced to an occasional temporary dissociation of AmpR from its binding site allowing transcription to occur. During induction a β-lactam molecule binds to a PBP affecting peptidoglycan metabolism. This results in the generation of a peptidoglycan fragment which may act as an autoinducer or a precursor for an autoinducer. This unidentified fragment is transported across the CM where it interacts with AmpR. Amp G and AmpE may also be involved in the process. AmpR is then prevented from blocking the transcription of the *ampC* gene and β-lactamase is produced.

For induction to occur there needs to be a sensor which "detects" the presence of the β-lactam inducer. In inducible systems the production of β-lactamase is triggered by the presence of β-lactam compounds (Citri and Pollock, 1966). Different β-lactam antibiotics differ in their ability to induce β-lactamase production. For example, imipenem and cefoxitin are very good inducers while aztreonam, ceftazidime and piperacillin are not (Sanders, 1983; Jacobs *et al.*, 1984; Livermore *et al.*, 1985; Graham and Medeiros, 1980; Hoffmann *et al.*, 1981; Cullmann and Dick, 1985). These compounds also differ in their affinity for penicillin binding proteins which are the only known targets for β-lactams in the cell. The ability of an antibiotic to induce β-lactamases and the degree to which it binds to PBPs may be related. That is, PBPs may be the cell's sensory system for the presence of β-lactams and it may be these which trigger the induction of β-lactamase synthesis (Oliva *et al.*, 1989).

Normark *et al.* (1990) have suggested the following sequence of events leads to β-lactamase induction: binding of a β-lactam to a PBP affects peptidoglycan metabolism, resulting in the generation of a peptidoglycan derived fragment which may act as the autoinducer or as an autoinducer precursor (Normark *et al.*, 1990). This unidentified fragment is transported across the CM where it interacts with AmpR. AmpG and AmpE may also be involved in the process. The β-lactam mediated effect in wild type cells is down regulated by AmpD. In *ampD* mutants, the autoinducer or its precursor is produced even in the absence of β-lactams. AmpD mutants show changed peptidoglycan composition and this suggests that *ampD* may usually regulate peptidoglycan synthesis. Addition of diaminopimelic acid induces β-lactamase production (Normark *et al.* 1990). It was suggested that AmpR "parasitises" on a normal regulatory system for peptidoglycan metabolism operating in enterobacteria (Normark *et al.*, 1990).

AmpG may be required to transduce a β-lactam induced stimulus across the CM or it could be involved in the transport into the cells of an autoinducer binding to AmpR. Another possibility is that AmpG may be involved in the generation of the autoinducer. Without ampG no induction or β-lactamase expression is possible. Everett *et al.* (1991) have found evidence that suggests β-lactams are sensed in the periplasm and that cytoplasmic components of the induction system are subsequently informed by signal transduction across the membrane (Everett *et al.* 1991). The derived amino acid sequence of *ampG* suggests that it is a trans-membrane protein. Mutants lacking the transduction mechanism encoded by *ampG* are unable to respond to a β-lactam stimulus

1.7 β-Lactamase action

Substrate profile has been used as a means of classification and characterisation of β-lactamases. β-Lactamases (E) function by first binding to the β-lactam substrate (S) in a non-covalent Michaelis complex (E.S), which may dissociate or undergo a hydrolytic reaction, forming an acyl-enzyme (E-S) (Knott-Hunziker *et al.*, 1980). Deacylation of this covalent complex and release of the ring-opened product (P) completes the reaction (Bush and Sykes, 1984; Fisher *et al.*, 1980).

β-Lactamases exhibit a wide range of substrate specificities and hydrolytic rates, e.g. *E. coli* TEM-2 β-lactamase catalyses the hydrolysis of as many as 2000 molecules

of benzylpenicillin per second per molecule of enzyme (Fisher *et al.*, 1980) but exhibits poor hydrolytic activity against many of the new β-lactam compounds; the hydrolysis of ceftazidime and cefoxitin proceeds at less than 0.1 molecule of β-lactam per molecule of enzyme per second (Bush and Sykes 1984). This turnover number, or k_{cat} value, is represented in the equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_3} E\text{-}S \xrightarrow{k_4} E + P$$

by k_4 (the rate constant for release of hydrolysed β-lactam) and is directly proportional to V_{max}. $V = V_{max}[S]/(K_m + [S])$ represents the rate at which a β-lactam antibiotic is hydrolysed. V is the measured velocity of hydrolysis, V_{max} is the maximum velocity attainable for the reaction, [S] is the substrate concentration, and K_m is the Michaelis constant (an equilibrium constant equal to the substrate concentration that will be hydrolysed at a rate equal to $0.5\ V_{max}$). K_m and V_{max} are kinetic characteristics that vary widely for different combinations of enzyme and substrates and may be determined by a number of standard diagnostic plots (Eisenthal and Cornish-Bowden, 1974; Mahler and Cordes, 1971).

Substrate profiles provide the basis for the study of β-lactam-β-lactamase interactions. Predictions may be made regarding the suitability of a particular antibiotic for use in the presence of β-lactamase producing bacteria from substrate profiles. β-Lactamases exhibit substrate inhibition at substrate concentrations greater than about 1 mM (Bush, 1983) so substrate profiles generated by using a single, high concentration of substrate (5 or 6 mM) (Jack and Richmond, 1970; Ross *et al.*, 1973) may be invalid. More recent studies have used lower, fixed concentrations of 100 μg/ml (about 200 μM) or 100 μM, both concentrations representing levels of antibiotic that may be either above or below K_m values for a range of common β-lactam antibiotics.

The best presentation of hydrolysis data for comparison purposes is with the ratio V_{max}/K_m. This ratio, termed "physiological efficiency" or "efficiency of hydrolysis", has been used to predict susceptibility of antibiotics to a variety of β-lactamases. If the K_m value for an antibiotic is low then the value of the ratio (V_{max}/K_m) may be high. Drugs with this pattern of behaviour may be destroyed rapidly under the conditions encountered in the periplasm (Bush *et al.*, 1985;

Livermore, 1985; Vu and Nikaido, 1985).

1.8 Catalytic pathway.

Until recently few data have been available to postulate a catalytic pathway for β-lactam hydrolysis. The reaction is analogous to peptide hydrolysis and the β-lactamases may have evolved, by analogy to the proteases, to utilise nucleophilic, electrophilic or general acid base catalysis involving serine, thiol, carboxyl or metal ion residues. So far only examples of serine (class A and C) and zinc (class B) enzymes have been found, although there is an example of a thiol β-lactamase and some β-lactamases are known to be sensitive to thiol reagents (Georgopapadakou and Sykes, 1983).

Experimental results are consistent with a covalent, acyl-enzyme (penicilloyl-enzyme) intermediate. The first evidence for a covalent intermediate in catalysis involved the poor substrate cefoxitin and *E. coli* TEM-2 enzyme (Fisher *et al.*, 1980). Cefoxitin possesses an α-methoxy group at C7 which results in very slow hydrolysis, allowing direct observation of the build up and decay of the putative acyl-enzyme intermediate. The nature of the covalent linkage was established by gel filtration following denaturation of the intermediate using radioisotope-labelled cefoxitin and Fourier transform infrared measurements (Fisher *et al.*, 1980).

Existence of a covalent intermediate was demonstrated in the reaction of β-lactamase I (from *B. cereus*) with a good penicillin substrate using either low pH or sub-zero temperatures to trap the intermediate formed in the reaction with dansyl-penicillin. A covalent acyl-enzyme intermediate was also shown using a good dansyl-labelled cephalosporin substrate and the enzyme from *S. aureus* PC1 (Anderson and Pratt, 1983; 1981). Detailed kinetic analysis of the reaction revealed that the rate of the reverse acylation step (reclosure of the β-lactam ring with expulsion of the enzyme) was approximately one third that of the corresponding forward (acylation) rate constant (Anderson and Pratt, 1981). This suggests that, in the acyl enzyme, the cephalosporinoyl moiety is maintained in a very similar conformation to that in the substrate, and that the acyl bond may be strained. Recent studies (Faraci and Pratt, 1984; 1985) have shown that in the β-lactamase-catalysed hydrolysis of cephalosporins with a good 3'-leaving group, the departure of the 3'-leaving group is not necessarily concurrent with β-lactam cleavage. Thus, two types of acyl-enzyme may be formed,

one containing the 3' leaving group and the other not. The latter has a very low rate of hydrolysis. Kinetic studies on the effect of pH and solvent kinetic isotope effects on β-lactamase I catalysis have been reported (Hardy and Kirsch, 1984).

Reactions of the class C enzymes from *P. aeruginosa* and *E. coli* K12 (the *ampC* chromosomal enzyme) with cloxacillin (Knott-Hunziker *et al.*, 1982) supports the idea of an acyl-enzyme intermediate. The results of the work indicate two significant mechanistic differences between the class A and class C enzymes. The former do not show transferase activity toward simple added nucleophiles such as hydroxylamine or methanol. However, the class C enzymes exhibit transferase activity towards alcohols, and also catalyse the hydrolysis of the resulting esters, such as α-methyl benzyl penicilloate ester (Knott-Hunziker *et al.*, 1982), i.e. they can function as esterases, which is analogous with the function of the serine proteases.

1.9 β-Lactamase inhibition

Hydrolysis does not always occur when a β-lactam binds to β-lactamase. A number of β-lactam molecules act as potent inhibitors, or inactivators, for a variety of β-lactamases. The mechanism for β-lactamase inhibition can be represented by equation 1 if S is replaced by I (inhibitor). This initial interaction is characterised by an equilibrium constant: $K_i = k_{-1}/k_1$. Other kinetic constants may also be defined, depending on the type of inhibition observed (Bush and Sykes, 1983; Cartwright and Waley, 1983; Knowles, 1985). With the development of β-lactamase stable β-lactams and novel β-lactamase inhibitors, much confusion has arisen with respect to methodology and terminology. β-Lactamase inhibitors may fall into several classes:

(a) metal ion chelators such as EDTA or ortho-phenanthroline (Sabath and Abraham, 1966) which inhibit the metalloenzymes,

(b) amino acid modifiers such as p-chloromercuribenzoate (pCMB) or the boronic acids (Beesley *et al.*, 1983; Cartwright and Waley, 1984; Jack and Richmond, 1970),

(c) active-site-directed irreversible inhibitors such as the suicide inactivators clavulanic acid (Brown, 1986; Fisher *et al.*, 1978) and sulbactam (English *et al* , 1978; Fisher *et al.*, 1981), and

(d) β-lactam antibiotics that have very low k_{cat} and K_m values, such as aztreonam,

moxalactam, and cefoxitin (Bush *et al.*, 1982; Bush *et al.*, 1985; Fisher *et al.*, 1980). Data obtained for each class of inhibitor reflect the features relevant to that molecule, e.g. suicide inactivators should exhibit time-dependent inhibition (Abeles and Maycock, 1976). β-Lactam antibiotics that are poor substrates should behave as classical competitive inhibitors with characteristic K_i values.

1.10 Inhibition pathway

There are two approaches to combating the problem of bacterial resistance. Firstly, the synthesis of β-lactams that are both resistant to the action of β-lactamases and effective and wide spectrum antibiotics. These are exemplified by the third generation cephalosporins e.g. moxalactam, cefuroxime (Maugh, 1981). Secondly, the synthesis of compounds that effectively inactivate β-lactamases, but have poor antibiotic properties and must be used in conjunction with a good antibiotic. Examples of compounds suitable for combination with β-lactamase susceptible β-lactams e.g. clavulanic acid (clavam) (Brown *et al*, 1976), sulbactam and tazobactam (penicillanic acid sulphone) (English *et al.*, 1978; Fisher *et al.*, 1981 Mezes *et al.*, 1982; Yamaguchi *et al.*, 1986). Of these clavulanic acid (as Augmentin, a mixture of amoxycillin and clavulanic acid, (Fuchs *et al.*, 1983)) and a combination of sulbactam and ampicillin are commonly used in therapy.

Enzyme inhibitors can be classified as either reversible or irreversible. Reversible inhibitors are those that bind to an enzyme in such a manner that the enzyme activity may subsequently be restored. The reaction between a β-lactamase (E) and a reversible inhibitor (I) may be represented by the equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I$$

This is a dynamic equilibrium and inhibition which may be diminished by diluting the inhibitor or by providing another molecule that binds to the enzyme at the same place as the inhibitor. Inhibitors of β-lactamases that bind at or close to the active site are often β-lactams. Competitive reversible inhibitors may be divided into two groups; competitive inhibitors which form E.I complexes but cannot form a reactive acyl-enzyme; and competitive or poor substrates which initially form reversible complexes, then an acyl-enzyme by a slow deacylation step and eventually a hydrolysis

product.

Another type of reversible inhibitor is a non-covalent inhibitor which binds at a site other than the active site of the enzyme. Non-competitive inhibition cannot be reversed by increasing substrate concentration unlike competitive inhibition which can be reversed by increasing the substrate concentration.

Reversible inhibitors may be characterised by an equilibrium constant, K_i, a value equal to the ratio of rate constants k_{-1}/k_1. K_i can be readily determined experimentally by a variety of kinetic methods. A K_i value is independent of substrate concentration and represents the affinity of the inhibitor for the enzyme.

Irreversible inhibitors may be more effective than reversible inhibitors in that the eventual result is destruction of enzymatic activity. Irreversible inhibitors usually require a finite time period in which to work. This time-dependence for inhibition is the result of the following series of reactions.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I \xrightarrow{k_2} E\text{-}I \xrightarrow{k_3} E\text{-}I^*$$

where E is the enzyme, I is the inhibitor, E.I is a reversible complex that can dissociate to free enzyme and inhibitor or can proceed to form a covalent complex (acyl-enzyme), E-I, and E-I* represents an inactivated enzyme that is not capable of processing substrate molecules.

Many compounds act as β-lactamase inhibitors, however, the mechanisms by which many of them function are not yet clear. The rest of this section will be devoted to a summary of the mechanisms as they are so far understood for the family of inhibitors which all share the common structural feature of a β-lactam ring.

The penicillanic acid sulphone sulbactam was first reported by English *et al.* (1978). It is a progressive, transient inhibitor of penicillinases or broad spectrum β-lactamases but is less effective against cephalosporinases. When used alone it has little antibacterial activity, however, when combined with β-lactam antibiotics against resistant strains that produce a susceptible β-lactamase, a synergistic effect is observed. Figure 2 rationalises the interaction of penicillanic acid sulphone with β-lactamase (Brenner and Knowles, 1981; 1984a; 1984b) This is the best understood mechanism so far and is most likely the one followed by any inhibitor which contains a 5-membered ring.

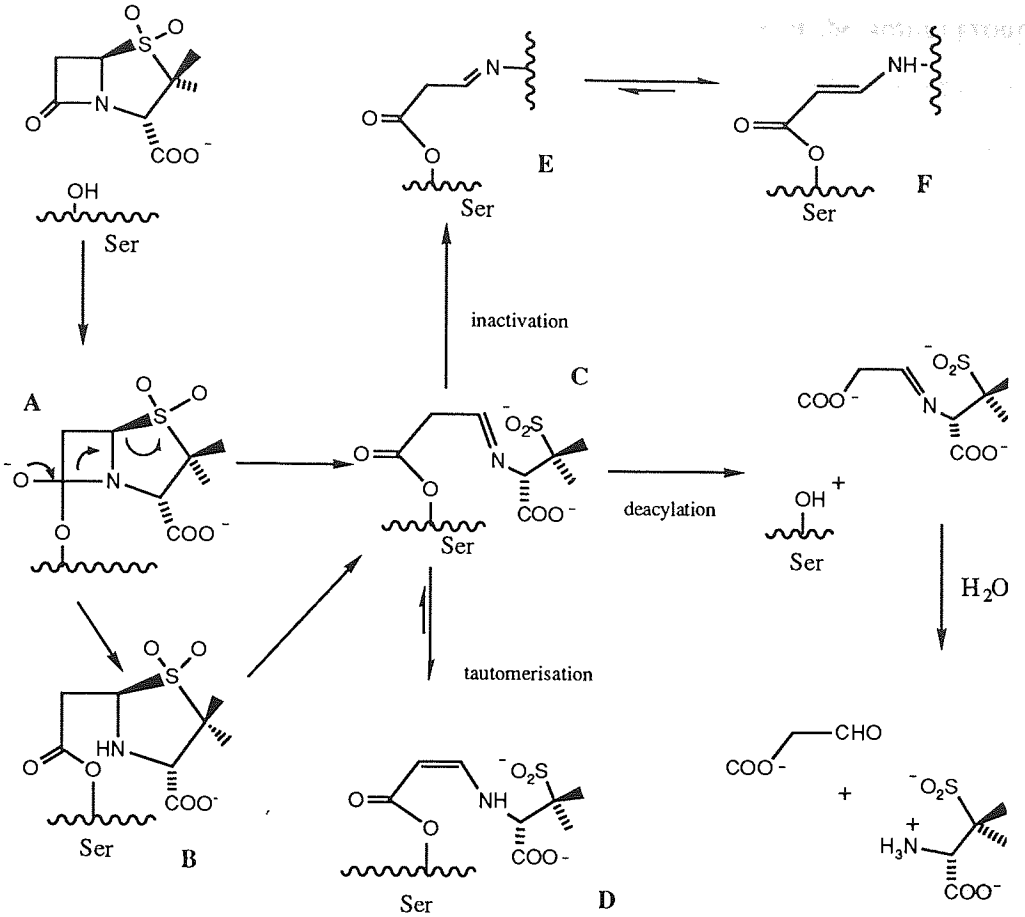


Figure 2. The interaction of penicillanic acid sulphone with a β-lactamase

In the first step of the reaction, the hydroxyl group of Ser 70 attacks the β-lactam carbonyl to yield a tetrahedral intermediate A. The intermediate (A) then either collapses immediately to form C (the imine acyl enzyme) or forms the imine acyl enzyme (C) in a step-wise fashion via an additional intermediate B. (This may account for the well known instability of the thiazolidine sulphones (Woodward *et al.*, 1949)). In the imine acyl enzyme both the β-lactam and the thiazolidine rings have been cleaved. The only difference between the normal penicilloyl-enzyme intermediate and the imine acyl-enzyme is that the oxidation of the thiazolidine sulphur to the sulphone makes the cleavage to the imine (step B to C) very easy. The imine acyl-enzyme C then deacylates and the imine hydrolyses to regenerate the native enzyme and two known products. Hence the inhibitor acts firstly as an enzyme substrate.

The imine acyl-enzyme C may also undergo tautomersation forming the more stable enamine form-the β-aminoacrylate ester (D) which represents a transiently inhibited form of the enzyme which may also regenerate the active enzyme. The imine

acyl-enzyme may also undergo transimination by the attack of the amino group of Lys 73 to give the imine (E) with the loss of the penicillamine part of the original sulphone (Brenner and Knowles, 1984a). The imine E could then undergo rapid tautomerisation to give the more stable enamine F which links Ser 70 and Lys 73 in the active site so irreversibly inhibiting the enzyme.

In 1979, Cartwright and Coulson reported that incubating the β-lactamase of *S. aureus* with 6-α-chloropenicillanic acid led to the inactivation of the enzyme. The mechanism of action already given for the sulphones may apply to this compound. Additional success have been observed with 6-β-acylaminosulphones. A 6-β-acylamino substituent makes the parent sulphide a poor enzyme substrate and potent β-lactamase inhibitor.

There is no indication that these additional penam sulphone derivatives pose any advantage over sulbactam. However, by studying the known mechanisms of action of these sulphones other compounds will presumably be identified, which may give rise to a novel inhibitor with therapeutic potential. Tazobactam is a new penicillanic acid sulphone which appears to be a very promising β-lactamase inhibitor. Its mechanism of action is most probably similar to that of sulbactam.

Clavulanic acid was first reported in 1976. The compound is weakly antimicrobial but being a potent β-lactamase inhibitor, it shows synergistic action against β-lactamase producing strains when combined with β-lactamase sensitive β-lactam antibiotics

The reaction of clavulanic acid with many different β-lactamases has been examined e.g. *E. coli* TEM (Charnas *et al.* 1978; Fisher *et al.*, 1978; Labia and Peduzzi, 1978), *S. aureus* PC1 β-lactamase (Cartwright and Coulson, 1979), *B. cereus* I enzyme (Durkin and Viwanantha,1978), and *Proteus mirabilis* and *K. pneumoniae* β-lactamases (Reading and Farmer, 1981). The most susceptible are the *S. aureus* and Gram-negative plasmid-mediated enzymes.

The mechanism of interaction of β-lactamase with clavulanic acid is analogous to the interaction of β-lactamase with the penicillanic acid sulphones (see Figure 3).

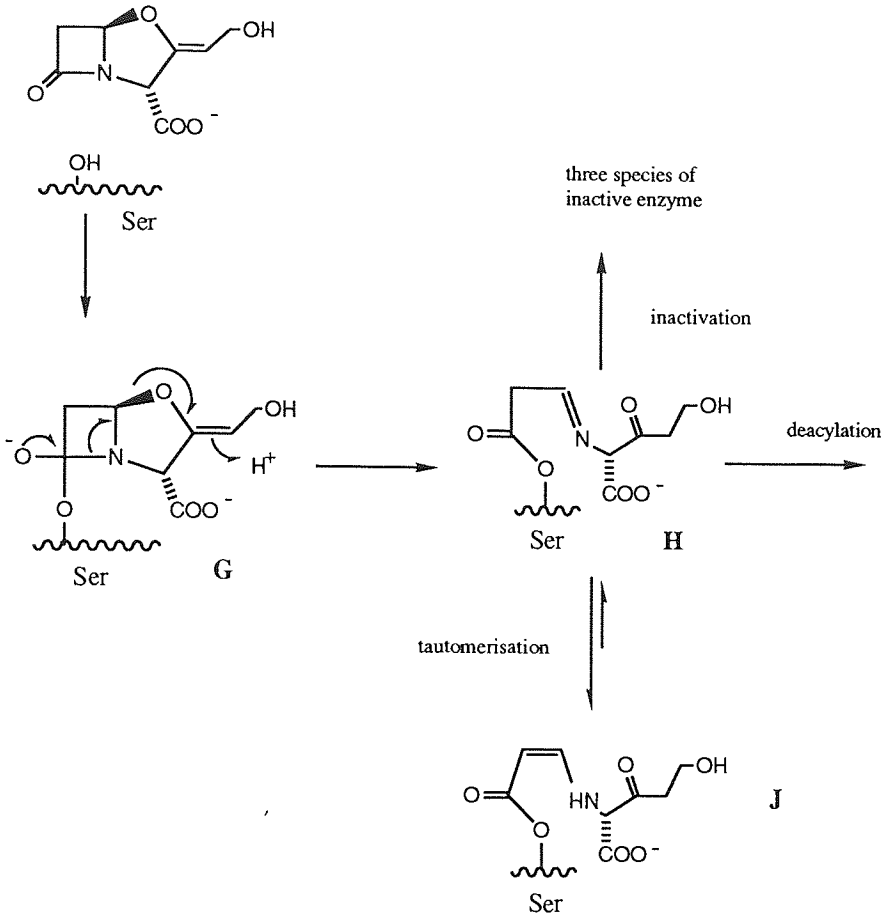


Figure 3. The interaction of clavulanic acid with TEM β-lactamase

The interaction of clavulanic acid with TEM β-lactamase leads to the formation of a tetrahedral intermediate (G). This then collapses with the opening of both β-lactam and oxazolidine rings to give the acyl-enzyme H. Like the easy conversion of A to C with the sulphones, the conversion of G to H is easy because of the ketonisation (in H) of the enol ether (in G). The acyl-enzyme H may tautomerise to the enamine J, the transiently inhibited form of the enzyme. Deacylation of H leads to malonsemialdehyde and the δ-hydroxy, β-keto, α-amino acid or products derived from them. The major difference between the reactions of sulphones and clavulanic acid is that clavulanic acid leads to 3 forms of inhibited enzyme whereas the sulphones lead to only one.

The side chain at C6 of the molecule may be important to stability. If the side chain makes clavulanate too easily recognisable, the deacylation rate may increase to compete with the acylation rate causing the process to break down. Many derivatives of the parent clavulanate molecule have been synthesised. Modification of the C6 side chain may increase the activity of the molecule towards those enzymes that resist the

parent compound.

The discovery of the carbapenems in 1976 has given rise to many interesting compounds, two of which are olivanic acid and theinamycin. The olivanates are best represented by the olivanic acids and epitheinamycins. In addition to showing antibacterial activity, olivanic acid is able to inhibit a wide range of β-lactamases, being more effective than clavulanic acid against isolated enzymes. The epitheinamycins are also though to be β-lactamase inhibitors but are clearly not as potent as the olivanic acids which might suggest that sulphation of the C8 hydroxyl group of the olivanates may increase antimicrobial potency and give rise to β-lactamase inhibitory properties.

Olivanic acid and epitheinamycin behave differently towards β-lactamases. During hydrolysis of epitheinamycin the simultaneous opening of the β-lactam ring and formation of the Δ^1 pyrroline occurs. Enzyme inactivation may also occur which may be due to the formation of an inactivating substance from the epitheinamycin.

During the interaction of olivanic acid with the β-lactamase (Figure 4) a tetrahedral intermediate is initially formed which rapidly converts to the acyl-enzyme Q. This then deacylates to the Δ^2pyrroline which can tautomerise to the thermodynamically more stable Δ^1 pyrroline (transiently inhibited enzyme) R.

In the case of epithienamycin the acyl-enzyme must be so short lived that the formation of transient enzyme inhibitor does not compete with deacylation. It seems that the carbapenems may only be β-lactamase inhibitors if the formation of the Δ^1 pyrroline competes with deacylation. Imipenem, which may react by an identical mechanism, is one example of a carbapenem currently in therapeutic use especially as an antipseudomonal.

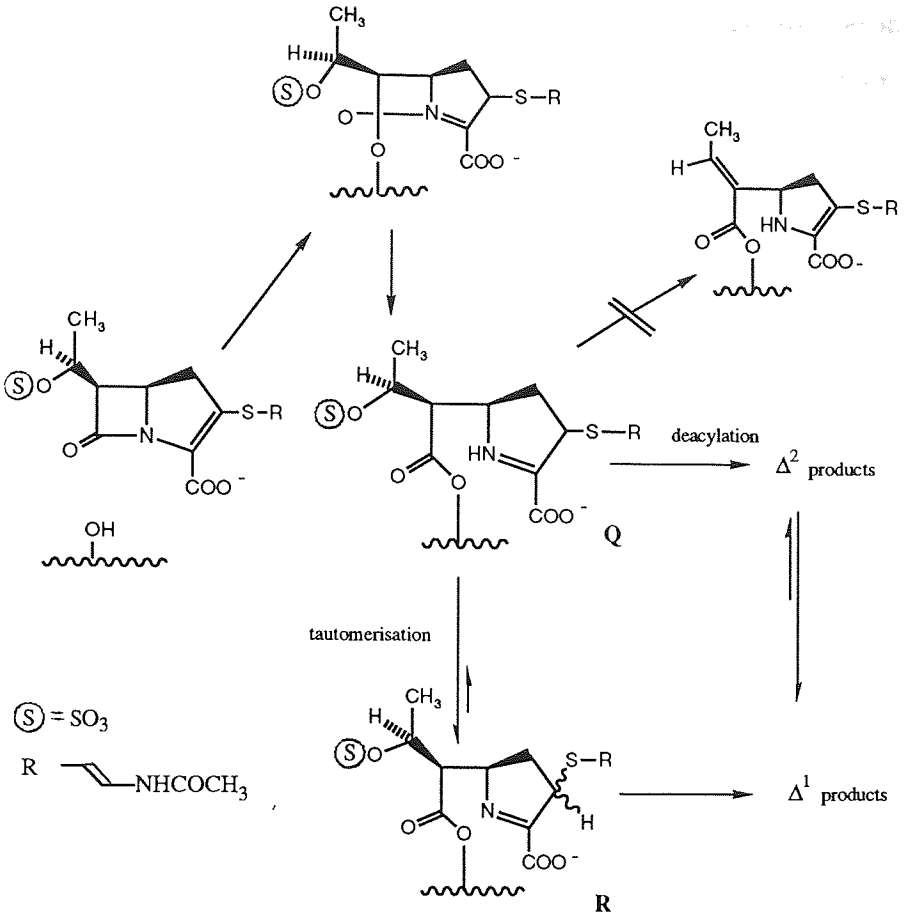


Figure 4. The interaction of olivanic acids with β-lactamase

Aztreonam is an example of a group of compounds known as the monobactams. It exhibits high antibacterial activity towards Gram-negative pathogens including *P. aeruginosa*, shows good stability towards β-lactamase and also acts as a β-lactamase inhibitor. The class A enzymes have poor affinities for aztreonam whilst class C cephalosporinases bind the inhibitor very tightly. Enzymatic studies have shown that aztreonam may form covalent enzyme-inhibitor complexes. The reaction product of the covalent complex with aztreonam is a simple cleaved β-lactam (Bush and Sykes, 1984; Bush and Smith, 1988). This may be the first step in a normal hydrolytic turnover (Bush *et al.*, 1985). Removal of excess inhibitor from the reaction mixture results in recovery of full enzyme activity.

Having an electron withdrawing substituent on the β-lactam nitrogen, a sulphonate in this case, allows the molecule to achieve these antibiotic properties as well as allowing recognition by β-lactamases. Stability to enzyme hydrolysis increased with the addition of an acidic oxime side chain in the monobactam and was enhanced further

by methyl substitution at the 4 position on the monobactam ring, as in aztreonam.

β-Lactam development is a continuing process and perhaps the advent of the fourth generation cephalosporins and other new β-lactam derivatives will go some way in reducing the threat posed by stably derepressed class C β-lactamases.

1.11 Identification of the active site residues of β-lactamases

Serine 70 has been shown to be the reactive serine in the active site of both class A and class C β-lactamases, using inhibition and radiolabelling studies. Ser 70 was implicated in β-lactam hydrolysis by studies of *B. cereus* β-lactamase inhibited with 6-β-bromopenicillanic acid (Pratt and Loosemore, 1978; Knott-Hunziker *et al.,* 1979a; Cohen and Pratt, 1980; Hill *et al.,* 1980). Inactivation of the enzyme with radiolabelled 6-β-bromopenicillanic acid followed by enzymatic digestion gave a labelled peptide fragment with the sequence FAFASTYK in which Ser 70 was covalently linked to the inhibitor (Hill *et al.,* 1980; Knott-Hunziker *et al.,* 1979; Cohen and Pratt, 1980). Subsequent investigations with other β-lactam inhibitors also demonstrated covalent attachment to this serine residue (Cartwright and Coulson, 1980; Fisher *et al.*, 1981; Kiener *et al.,* 1980). The active site sequence of the *P. aeruginosa* chromosomal class C enzyme was shown to be VTPETLFGIGSVSK after sequencing of the tryptic digest of the cloxacillin labelled fragment after affinity labelling with cloxacillin. With radioactive cloxacillin, a penicilloyl-serine at the active site of the acyl-enzyme was identified (Knott-Hunziker *et al.*,1979b; 1982). Separation of excess inhibitor by gel filtration under denaturing conditions followed by freeze drying and digestion with trypsin yielded a single, 14 residue long radioactive peptide. Subsequent thermolysin digestion yielded smaller fragments, one of which (IGS) contained the only amino acid with a potentially reactive side chain (Knott-Hunziker *et al.*, 1982). The active site sequence of the *E. cloacae* P99 chromosomal β-lactamase was shown to be LFELGSISK using 6-β-iodopenicillanate (Joris *et al.,* 1984) and that of *C. freundii* was shown to be GSxSK using dansyl penicillin (Joris *et al.*, 1986). In each case Ser 70 was identified as the reactive residue. Sequence comparisons reveal a common structural motif, SxxK (Joris *et al.,* 1984; Ghysen *et al.*, 1980; Waxman *et al.,* 1982). This evidence supports the idea that the SxxK motif must be important for the

mechanism and suggests that Ser 70, which is always conserved, could be the key active site residue.

Site specific mutagenesis of the pBR322 RTEM enzyme from *E. coli* has been used to identify active site residues. Replacement of Ser 70 by cysteine resulted in reduced catalytic activity (Sigal *et al.*, 1982), sensitivity to thiol reagents (previously not shown) and differing substrate activities to that of the wild type, replacement with Lys reduced catalytic activity (Sigal *et al.*, 1984), whilst replacement with threonine resulted in complete loss of catalytic activity as did replacement of Thr 71 with serine (Dalbadie-McFarland *et al.*, 1982).

Structural analysis using X-ray diffraction crystallography has been carried out on a number of β-lactamases: *B. cereus* I (Aschaffenberg *et al.*, 1978), *S. aureus* PC1 (Moult *et al.*, 1985), *B. licheniformis* (Dideberg *et al.*, 1985) and *E. cloacae* P99 (Charlier *et al.*, 1983). Full X-ray crystal coordinates have been determined for *C. freundii* (Oefner *et al.*, 1990), *S. aureus* PC1 (Hertzberg and Moult, 1987) and *Streptomyces albus* G (Dideberg *et al.*, 1987) β-lactamase, although only those of the PC1 are currently available (Brookhaven Data Base). Structural analysis of the DD-carboxypeptidase from *Streptomyces* R61, a possible relative of β-lactamases (Waxman *et al.*, 1982) has located a serine, homologous to Ser 70, and a conserved lysine in the penicillin-binding site in the 2.8Å resolution map (Bartolene *et al.*, 1984). The conserved Ser 70, Lys 73, Lys 321, Thr 322 and Gly 323 in the crystal structure of the *C. freundii* β-lactamase all lie around the active site, supporting the idea that these residues are involved in catalysis, substrate binding, or both. The sequences of β-lactamases and PBPs all contain a conserved Lys at position 73 which suggests a vital function for this residue. Support for the role of Lys 73 in catalysis comes from several experiments: modification of free amino groups in *S. aureus* β-lactamase by methyl acetimidate leads to 99% inactivation (Pain and Virden 1979) but inactivation is prevented by the presence of cephaloridine; conversion of Lys 73 to Ser in *E. coli* RTEM leads to a reduction in activity; whilst studies using the irreversible β-lactamase inhibitors phenylpropynal (Schenkein and Pratt, 1980) and phenylglyoxal (Borders *et al.*, 1982) give results consistent with the presence of lysine or arginine in the active site vicinity; mutation of Lys 73 to Arg reduced activity 100-fold in *B. cereus* 569/H.

Hertzberg and Moult (1987) suggest the role of Lys 73 in *B. cereus* 569/H is to orient the proton leaving the Ser 70 and to facilitate its transfer to the β-lactam nitrogen. Lys 73 also makes a salt bridge interaction with Glu 166, deprotonates the serine hydroxyl group on acylation, and so has a role in acylation analogous to that in deacylation.

Lys 234 may also be involved in the catalytic mechanism. Mutation of Lys 234 to Glu or Ala in the β-lactamase of *B. licheniformis* had no effect on gross structure although a decrease in pI occurred because of the change from the positively charged ammonium group of lysine to a negatively charged carboxylate group of glutamate or neutral alanine. This suggested the effects on catalysis were not due to changes in protein conformation. The effect of pH on thermal stability was explained by electrostatic effects above pH 7.0, conversion of ammonium to carboxylate in the mutant led to destabilisation, possibly due to unfavourable electrostatic interactions with Glu 166. At low pH the mutant became relatively more stable, due to removal of an unfavourable interaction with Lys 73 in the wild type. This shows the importance of measuring these properties at more than one pH, especially when stabilising ionisable groups are present. If Lys 234 is involved in substrate binding the K_s of the enzyme substrate binding would change. Lys 234 is unlikely to contribute to the substrate ground-state binding but it could contribute to the transition state. The increase in the free energy barrier of the mutant suggests the deletion of a side chain which forms a H-bond with a charged group on the substrate (Ferst *et al.*, 1985). This energy value is less for the ala mutant which shows ala lacks H-bonding potential.

Sequence analysis of related enzymes, chemical modification experiments, and recent structural data, suggest the importance of a number of groups to catalysis in class A β-lactamases (Hertzberg and Moult, 1987). Ser 70 acts as a nucleophile in attacking the β-lactam carbonyl to form a covalent penicilloyl enzyme intermediate (acyl-enzyme). Lys 73 (of SxxK) might help to stabilise the oxyanion tetrahedral intermediate (Ellerby *et al.*, 1990; Carroll and Richards, 1990) or may help the transfer of a proton from Ser 70 to the β-lactam nitrogen (Moews *et al.*, 1990). Glu 166 might help the transfer of protons in the acylation and deacylation steps (Madgwick and Waley, 1987) and Lys 234 possibly acts as an electrostatic anchor for the substrate carboxylate (Ellerby *et al.*, 1990) based on analogy with residues in R61 DD-peptidase (Kelly *et al.*, 1986).

1.12 *P. aeruginosa*

P. aeruginosa is a motile, non-spore-forming, aerobic, Gram-negative rod capable of producing diffusible fluorescent pigments and a soluble phenazine pigment, pyocyanin. It can be isolated from soil and water but is commonly isolated from wounds, burns, urinary tract, respiratory tract, infections and immunocompromised patients. Its clinical significance stems from its high degree of resistance to antimicrobial compounds, (due to the production of degradative and modifying enzymes); the diffusion barrier or reduced penetration of the drug to the target enzymes (due to the low cell wall permeability); mutations of the target enzymes; exopolysaccharide products; and other virulence factors.

1.13 Occurrence of *P. aeruginosa* in infection

P. aeruginosa is an opportunistic pathogen which readily colonises and infects the body surfaces of humans predisposed because of immunological defects, CF, immune suppression following transplants, or alterations in body surfaces such as burns or wounds. Infections commonly occur in hospitalised patients, intensive care or burns units and CF centres, where the organism may be endemic and represent an important cause of morbidity and mortality in these patients. Such patients have multiple risk factors i.e. compromised host defences, respiratory therapy support, vascular access devices, urinary catheterisation, prolonged hospital stay and heavy exposure to antibiotics (Follath *et al.*, 1987; John *et al.*, 1982; Sanders *et al.*, 1982; Cryz, 1984; Bodey *et al.*, 1983) which makes them highly susceptible to infection.

P. aeruginosa produces a number of virulence factors which may play a role in human disease, either alone or in combination (Woods and Iglewski, 1983; Que and Woods, 1987; Nicas and Iglewski, 1986). The surface components of the organism (slime layer and endotoxic lipopolysaccharide) have antiphagocytic functions and interfere with the mucocilliary blanket in removal of the organisms from the respiratory tract (Pier and Elcock, 1984); these factors help the survival and the establishment of the organism in the host (Costerton *et al.*, 1987). Leukocidin may prevent phagocytosis and contribute to establishment in the host. Exotoxin S inhibits protein synthesis in the host (Clarke, 1990; Woods and Sokol, 1985; Nicas *et al.*, 1985). Shock is frequently associated with *P. aeruginosa* infection and may be due to the lipopolysaccharide (LPS)

and the heat labile exotoxin A (Nicas and Iglewski, 1986). Exotoxin A produces dermonecrotic lesions, a drop in circulating leukocytes, inhibits protein synthesis and may result in death (Pavloviskis *et al.*, 1978; Iglewski *et al.*, 1977; Iglewski *et al.*, 1978). Protease, elastase, collagenase and exotoxin may cause haemorrhagic necrotic lesions in infections of the skin, eyes and internal organs (Woods *et al.*, 1982; Walker *et al.*, 1978). The glycolipid haemolysin and phospholipase C haemolysin may be involved in pulmonary disease; destroying the lung surfactant and causing atelectasis and necrosis (Cash *et al.*, 1982). A heat-labile substance, probably an enterotoxin, in conjugation with other enzymes and toxins, may be responsible for the necrosis and fluid loss associated with a necrotizing enteritis which occurs in humans (Nicas and Iglewski, 1986).

1.14 Aims of the study

P. aeruginosa continues to be a serious clinical problem, especially in CF patients where the selection of derepressed β-lactamase producing strains has caused outbreaks of β-lactam resistant lung infections (Giwercman *et al.*, 1990). The expression of some virulence factors has been shown to be influenced by environmental iron concentrations (Bjorn *et al.*, 1978, 1979; Sokol *et al.*, 1982). Iron depleted conditions have been shown to exist in the lung (Brown *et al.*, 1984) and there may be a link between the increased expression of β-lactamase shown by some strains isolated from the lung and the iron depleted conditions fond to exist there. Some sucess in treating these resistant infections has been obtained by using a combination of a β-lactamase inhibitor with the β-lactamase sensitive β-lactam. One promising new inhibitor is tazobactam which has been found to have activity against class C β-lactamases (Micetich *et al.*, 1987; Higashitani *et al.*, 1990).

The aims of this study were:

(a) to isolate and investigate the kinetic properties of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}, investigate its inhibition kinetics in comparison with the well-characterised β-lactamases of *S. aureus* PC1 and *E. cloacae* P99.

(b) to examine the effects of growth conditions, especially iron availability on β-

lactamase production in *P. aeruginosa* isolates.

(c) to investigate the cellular location of the β-lactamase.

(d) to determine the properties and effectiveness of a new β-lactamase inhibitor tazobactam.

(e) to predict an active site structure for the β-lactamase and to identify candidate residues which interact with the inhibitor, tazobactam, and determine the structural features responsible for its enhanced activity over other inhibitors.

2. MATERIALS AND METHODS

2.1 Strains

Clinical isolates of *P. aeruginosa* (3, 10, 31, 54, 71, 75, 113, 177, 236, 241 and 258) were obtained from the sputa of seven patients (three female and four male, age 12-26 years) with CF during a two week course of anti-pseudomonal β-lactam antibiotic therapy at the Rigshospitalet, Copenhagen. Two reference strains were used: NCTC 6750 and PAO1 (ATCC 15692); and three mutants derepressed in expression of β-lactamase: PAO1 DR (a spontaneous derepressed mutant selected on carbenicillin agar by Dr. Julia Lodge, University of Birmingham, U.K.); 4082 (Matsumoto and Terawaki, 1982) and SAI^{const} (Curtis *et al.*, 1981). All strains were shown by isoelectric focusing to express only the chromosomal β-lactamase. Cultures were maintained on nutrient agar slopes and in 20% glycerol suspensions in liquid nitrogen.

2.2 Chemicals

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd., (Poole, Dorset), Sigma Chemical Company (Poole, Dorset) and Fisons (Loughborough, Leics.) and were of Analar grade or equivalent. The antimicrobial compounds used in this study were penicillin G (Sigma), ampicillin (Sigma), tazobactam (Lederle, Pearl River, USA), clavulanic acid (SmithKline Beecham, Brockham Park, Surrey) and sulbactam (Pfizer, Sandwich, Kent).

Purified β-lactamases of *E. cloacae* P99 and *S. aureus* PC1 were obtained from Cam-R.

2.3 Preparation of glassware to reduce the iron content

All glassware was fully immersed in 0.01% ethylenediaminetetraacetate (EDTA) and allowed to stand overnight at room temperature. It was rinsed ten times in single distilled water, then rinsed ten times in double distilled water before drying at 60°C. All glassware was sterilised at 121°C for 20 min.

2.4 Chemically defined medium

The composition of the chemically defined growth medium for *P. aeruginosa* formulated by Noy, 1982) is shown in Table 3. Extrapolation of the graph of culture OD_{470} against time showed that the concentration of each essential nutrient was sufficient to allow exponential growth to an optical density (OD) of 10, in practice however, oxygen became growth rate-limiting before any of the medium constituents. Iron depletion was achieved by growing the organism in CDM from which iron had been omitted (CDM-Fe); iron-replete bacteria were grown in CDM-Fe supplemented with 10 μM $FeSO_4$ (acidified with 1 μl/ml H_2SO_4 conc.) (CDM+Fe). The constituents were dissolved in the appropriate volume of double distilled water and sterilised by autoclaving at 121°C for 20 min. Glucose and iron were autoclaved separately and aseptically added to the medium.

Table 3. Composition of CDM for *P. aeruginosa* strains.

Constituent	Final Concentration (mM)
KCl	0.62
NaCl	0.5
$K_2HPO_4.3H_2O$	3.2
$(NH_4)_2SO_4$	40.0
$MgSO_4.7H_2O$	0.4
MOPS *	50.0
Glucose	40.0
$FeSO_4.7H_2O$†	0.01

*MOPS (4-morpholino-propane sulphonic acid) was adjusted to pH 7.4 with 10 M NaOH

† $FeSO_4.7H_2O$ (10mM) solution was acidified with 1μl concentrated H_2SO_4 per ml to prevent precipitation when autoclaving.

2.5 Complex media

Nutrient agar, nutrient broth and tryptone soya broth (TSB) were obtained from

Oxoid (Basingstoke) and *Pseudomonas* isolation agar (PIA) from Difco (West Molesey, Surrey).

2.6 Batch culture

Batch culture was carried out in Erlenmeyer flasks containing not more than 20% of their volume of appropriate media. Cells were grown in pre-warmed CDM (see section 2.4, Table 3) at 37°C and agitated at 180 rpm on an orbital shaking incubator. Growth of bacteria was measured by determining changes in OD_{470} of the culture with time. Samples for estimation of OD_{470} were removed aseptically at appropriate intervals, dilutions being made in fresh growth medium when necessary. Undiluted samples were returned to the flask to prevent undue reduction in volume, diluted samples were discarded. Changes in cell concentration during bacterial growth were followed using spectrophotometric measurement. At low cell concentrations the light scattered by a bacterial cell suspension is directly proportional to the cell concentration in the suspension, as expressed by the Beer-Lambert Law. This relationship is obeyed between an optical density (OD) of 0.03 and 0.3 (Kenward, 1975). Measurements of absorbance were made at a wavelength of 470 nm (OD_{470}) to minimise absorption by medium constituents and bacterial metabolic products such as pyocyanin. An OD of 1.0 at 470 nm indicated a concentration of approximately $1x10^9$ $cfu.ml^{-1}$ (Anwar, 1981).

2.7 Determination of the minimum inhibitory concentration (MIC) of antibiotics

Aliquots of double strength CDM (2.4 ml) were dispensed into test-tubes with 0.5 ml glucose (0.4M) and, for CDM+Fe conditions, 50 µl iron (10mM $FeSO_4$). Amounts of sterile antibiotic solution and distilled water were added to give the desired concentration in a 3 ml volume and finally a 100 µl inoculum (diluted in CDM to give $1x10^6$ $cfu.ml^{-1}$) from an overnight culture was added. The tubes were vortex-mixed and incubated at 37°C for 18 h. The tubes were examined for growth and the MIC defined as the lowest concentration of antibiotic that inhibited the development of visible growth.

2.8 Isolation and purification of β-lactamase

2.8.1 Ammonium sulphate precipitation. The cells were grown in a variety of media: nutrient broth, nutrient broth supplemented with penicillin G (500 μg/ml); CDM-Fe; CDM+Fe; CDM-Fe supplemented with penicillin G (500 μg/ml); and CDM+Fe supplemented with penicillin G (500 μg/ml); all at 37°C in an orbital shaking (180 rpm) incubator. Cells were harvested at 18000 *g* for 10 min, washed twice in 0.85% saline and resuspended to a final volume of 20 ml in sterile distilled water. Aliquots (5 ml) of the cell suspension were made and the cells broken by 6 x 30 s pulses of sonication (0.5 cm diameter probe operating at full power, MSE Soniprep) with 30 s intervals for cooling in an ice bath. A small aliquot (1 ml) of the cell homogenate was retained for assay. Whole cells and cell envelope material were removed by centrifugation at 48500 *g* for 20 min. The supernatant was retained for assay.

Solid ammonium sulphate was added to the supernatant to give 30% saturation. The suspension was mixed continuously for 15 min at 4°C until all the ammonium sulphate had dissolved. The suspension was then centrifuged for 10 min at 18000 g. The pellet was retained, redissolved in 2 ml of sterile distilled water and then dialysed against sterile distilled water for 18 h at 4°C. The precipitation procedure was repeated on the supernatant increasing the saturated ammonium sulphate concentration to of 60% saturation. The suspension was again centrifuged at 18000 *g* for 10 min and the pellet resuspended in 2 ml sterile distilled water and dialysed as before. The ammonium sulphate concentration was then increased to 90% saturation and the procedure repeated. The final 90% supernatant and the pellets obtained at each precipitation stage were dialysed against distilled water for 18 h at 4°C. This completed the first stage of the purification process. After dialysis the fractions were then assayed for protein and β-lactamase activity.

In the second stage of the purification process the 60 and 90% dialysed precipitation fractions were recombined and the precipitation procedure repeated using 30, 40, 50, 60, 70, 80 and 90% ammonium sulphate saturated solutions. The pellets and 90% supernatant were again dialysed for 18 h at 4°C. The dialysed fractions were then assayed for protein and β-lactamase activity. The amount of solid ammonium sulphate to be added to a solution already at saturation S_1% to take it to a saturation of

S_2% (Scopes and Stoter,1982) was calculated from :

$$\text{Weight (g)} = 533(S_2-S_1) / (100-0.3S_2)$$

2.8.2 Fast protein liquid chromatography (FPLC). The gel filtration chromatography column used was Pharmacia Superose 12 HR 10/30 (cross-linked agarose; optimum separation range molecular weight 1000 - 3 x 10^5; average particle size 8-12 μm; column length 30 cm, diameter 1.5 cm). All buffers and solutions were filtered through Millipore or Gelman acrodisc 0.22 mm cellulose acetate filters and degassed under vacuum. Samples were centrifuged in a microcentrifuge at 13000 rpm for 5 min to remove particulate material. Prior to use, the column was equilibrated with at least 2 volume changes of buffer (potassium phosphate, 5 mM, pH 7.0). Samples were applied by loop injection (500 μl) to the top of the Superose 12 column and pumped through with buffer at a rate of 36 ml/h using a Pharmacia FPLC system with P-500 pump. Fractions were collected and assayed for β-lactamase activity and protein content and used for IEF, SDS-PAGE and kinetic studies.

Molecular weight was determined by comparison to the retention time of the sample by the column and the retention time of a number of known molecular weight markers. The markers used were: cytochrome c, 12.3 kD; carbonic anhydrase, 29 kD; lactate dehydrogenase, 36.4 kD; ovalbumin, 48 kD; bovine serum albumin, 66 kD; fructo-6-phosphate kinase, 84 kD; and β-galactosidase, 116 kD.

2.8.3 Isoelectric focusing (IEF). Analytical IEF was performed using an LKB 2117 Multiphor system according to the method of Matthew *et al.*, (1975). Polyacrylamide gel (12 cm x 9 cm x 1 mm) was cast on a thin glass plate (1 mm). The composition of the gelling mixture is given in Table 4.

Enzyme samples were loaded onto the gel using small rectangular pieces of filter paper (Whatman 3 mm Paratex, LKB) placed 1 cm from the anode. Each loading contained approximately 100 μg protein. All experiments were performed at 25 W constant power and at 10°C. After 1 h the sample loading strips were removed and the run continued for a further 1-2 h. β-Lactamase was detected using nitrocefin (0.025 mg/ml solution). A nitrocefin-soaked filter paper was laid over the gel and the distance

from the anode to the first pink-coloured band(s) detected was measured. The pink bands were formed by the hydrolysis of nitrocefin by the focused β-lactamase. Alternatively, the gel was stained for protein by first fixing the gel in a solution of 10%

Table 4. Composition of analytical IEF gel.

Component	Volume (ml)
acrylamide (100 g) + methylene bis-acrylamide (2.7 g) in 300 ml water	2.7
tetramethyl-ethylenediamine (TEMED) (5% soln. in distilled water)	0.06
ampholine w/v 40% various pH ranges	
pH 3-10	0.2
pH 5-8	0.4
distilled water	8.3

After mixing, 1.3 ml of riboflavin (2 mg/100 ml) was added to initiate polymerisation. Commercially prepared gels (LKB, pH range 3.5-10) were also used. Electrode Solutions: anode, 1 M H_3PO_4; cathode, 1 M NaOH.

trichloroacetic acid for 30 min then immersing the gel in Coomassie blue R-250 for 30 min and destaining in methanol/acetic acid (30/10% mixture) to remove excess stain. Marker proteins (LKB) of known pI values were used to plot a calibration curve of pI versus distance moved from the anode (mm).

2.8.4 Preparative isoelectric focusing. Preparative or block IEF was carried out in a semi-solid Sephadex G75 bed mounted on an LKB 2117 Multiphor system. The pre-swollen slurry (3 g Sephadex in 30 ml water + 1 ml ampholine, pH 3.5-10) was poured into a horizontal rectangular glass mould (20 cm x 10 cm x 0.5 cm) and the surface moisture removed by sprinkling dry sepharose powder over the surface of the slurry. The sample was applied by means of a small well made in the slurry. Filter

paper wicks soaked in the appropriate electrode solution were then laid so as to make contact between the sepharose block and electrode compartments. β-Lactamase samples (1 ml) were applied to one end of the gel (1 cm from the cathode) using a Gilson pipette. The sample was allowed to soak into the gel. The separations were carried out overnight at a constant current of 25 mA.

β-Lactamase activity was then located by inserting the edge of a strip of filter paper previously soaked in nitrocefin (0.025 mg/ml) into the Sephadex and noting the position of any pink band which might develop. The region of sepharose corresponding to the pink band was then excised and the enzyme eluted from the gel. The excised sepharose slurry was packed into a short plastic column (10 cm x 2.5 cm diameter) with the end sealed by 1 cm depth of glass wool. Phosphate buffer was then poured onto the top of the column and the outward flow adjusted, by means of a clamp, to 2 ml min^{-1}. The eluent was collected in a microtitre plate in 250 µl aliquots. These fractions were then assayed for β-lactamase activity by the method given in section 2.12.1. Although the method was intended for preparation of the enzyme it proved to be a valuable analytical method.

2.8.5 Freeze drying of purified β-lactamase. Fractions collected from FPLC gel filtration chromatography which gave a single protein band on SDS-PAGE were pooled and then freeze dried. 5 ml of the pooled protein solution were placed in a 30 ml plastic universal bottle and flash-frozen in liquid nitrogen. After freezing the bottle was then capped with perforated parafilm and placed in a lyophilisation flask. The flask was then connected to an Edwards freeze drier under vacuum until the contents of the tube had dried to a solid.

2.9 Protein assay

2.9.1 Lowry protein assay. The protein contents of ammonium sulphate fractionations and OM preparations were determined by the method of Lowry *et al.*, (1951) and Peterson (1977), the latter being a simplification of the Lowry assay. Bovine serum albumin (BSA) standards (0-300 µg) and the samples for analysis were made up to 0.5 ml with double distilled water. A reagent blank containing 0.5 ml

double distilled water was also prepared. An equal volume of 1M NaOH was added to each sample and the reagent blank and heated to 100°C for 5 min. On cooling, 2.5 ml of a solution containing 1 ml 0.5% w/v $CuSO_4.5H_2O$ and 1 ml 1% w/v NaK tartrate solution in 50 ml of 5% w/v Na_2CO_3 in 0.1M NaOH was added. After leaving for 10 min, 0.5 ml Folin-Ciocalteau reagent diluted 1:1 with distilled water was added to each sample and the blank and mixed by vortexing. The OD at 750 nm was recorded after 30 min against the reagent blank and a calibration curve constructed. Standards and samples were assayed in triplicate.

2.9.2 Protein profile determination. Membrane samples were separated by sucrose density gradient centrifugation. Fractions (0.25ml) were collected in a microtitre plate after piercing the base of the tube with a syringe needle. Membrane bands (proteins) were detected by measuring the absorbance of the fraction at 340 nm in an Anthos plate reader. The absorbance of each fractions at 340 nm was then plotted against fraction number to give the membrane protein profile.

2.10 β-Lactamase activity

2.10.1 Determination of enzymes' specific activities. Specific enzyme activity was determined using the method of O'Callaghan *et al.*, (1972) and was carried out in a Pye-Unicam SP6-400 UV spectrophotometer and a Euroscribe strip chart recorder (Gallenkamp). Stock solutions of 5 mM potassium phosphate buffer pH 7.0 and nitrocefin (0.025 mg/ml) were pre-warmed to 37°C. 0.1 ml of stock nitrocefin and 0.8 ml of phosphate buffer were mixed in a 1 cm cell with a total volume of 1 ml, which was then placed in the spectrophotometer. 0.1 ml of the crude β-lactamase preparation (or an appropriate dilution in phosphate buffer) was added and mixed thoroughly. The chart recorder (speed 1 cm/min) recorded the initial rate of hydrolysis as the appearance of the red hydrolysed product at nitrocefin at 492 nm. Each rate was determined 5 times to obtain a mean value and used to calculate a specific activity. To determine the kinetic parameters, the β-lactamase assay was miniaturised and carried out in an Anthos plate reader.

2.10.2 Enzyme profile determination. Membrane samples were separated by sucrose density gradient centrifugation. Fractions (0.25 ml) were collected in a microtitre plate after piercing the base of the tube with a syringe needle. 20 µl samples of each fraction were placed in a microtitre plate. 80 µl of 5 mM phosphate buffer (pH 7.0) were added to each fraction sample and the reaction initiated by the addition of 50 µl of a 0.025 mg/ml solution of nitrocefin. The plate was then incubated at 37°C for 2 min and the absorbance of each sample at 492 nm measured. The absorbance of each fraction at 492 nm was then plotted against fraction number to give the membrane enzyme profile.

2.10.3 Determination of reversible or irreversible character of enzyme inhibition. The irreversible or reversible natures of the inhibitions was investigated for the *P. aeruginosa* SAI^{const}, *S. aureus* PC1 and *E. cloacae* P99 β-lactamases. The kinetic assay from 2.13.3 was repeated for just one substrate and inhibitor concentration. The pre-incubation time of enzyme and inhibitor was varied over the range of 0-160 min. The reaction was then initiated by the addition of nitrocefin and the rate of change of absorbance monitored over 10 min at 492 nm. The length of the lag period before the start of the reaction was then measured and plotted against pre-incubation time. V_{max} was also calculated for the reactions.

2.10.4 Determination of reversible or irreversible character of enzyme inhibition using V_{max} and E_o method. The irreversible or reversible natures of the inhibitions was investigated for the *P. aeruginosa* SAI^{const}, *S. aureus* PC1 and *E. cloacae* P99 β-lactamases. A range of enzyme concentrations were pre-incubated with a fixed concentration of tazobactam. The reaction was then initiated by addition of a fixed concentration of substrate (nitrocefin). The reaction rate was then measured by measuring the change in absorbance at 492nm. The procedure was repeated for several different concentration of tazobactam. The V_{max} for each reaction was calculated and plotted against enzyme concentration (E_0).

2.10.5 Determination of inhibition kinetics. The inhibition kinetics of *P. aeruginosa* SAI^{const}, *S. aureus* PC1 and *E. cloacae* P99 β-lactamases were investigated with ampicillin, cloxacillin, cefotaxime and tazobactam. Initial experiments were conducted to determine suitable enzyme, substrate and inhibitor concentrations for use in the kinetic assays. Kinetic assays were carried out in microtitre plates which had been prepared by washing twice in 10% teepol solution, rinsed three times in single distilled water and twice in double distilled water. Five different substrate and inhibitor concentrations were investigated at a fixed enzyme concentration. 20 µl of the appropriate dilution of enzyme were pre-incubated for 5 min with 50 µl of the appropriate inhibitor dilution in 50 µl of 5 mM potassium phosphate buffer (pH 7.0) at 37°C in an Anthos plate reader. The reaction was then initiated by the addition of 80 µl of a 0.025 mg/ml solution of nitrocefin. The change in absorbance at 492 nm was then followed over 5 min and the V_{max}, K_m and K_i for each reaction calculated using Eadie-Hofstee and Dixon plots.

2.11 Preparation of bacterial OMs

2.11.1 Sarkosyl method. OMs were prepared by the method of Filip *et al.*. (1973). Cells were grown under various conditions at 37°C in an orbital shaking incubator and harvested by centrifugation at 5000 g for 10 min at 4°C. The resulting pellet from 500 ml of culture was washed twice in 0.85% saline and then suspended in 10 ml sterile distilled water. Cells were broken by 6 x 30 s pulses of sonication in an ice bath with 30 s intervals for cooling (maximum power, 1 cm diameter probe, MSE Soniprep). Sarkosyl (N-lauroyl sarcosinate, sodium salt) was added to a final concentration of 2% v/v to solubilize the cytoplasmic membrane and leave the OM intact (Lambert and Booth, 1982). After incubation at room temperature for 10 min, any remaining unbroken cells were removed by centrifugation at 5000 x *g* for 10 min. The supernatant was then centrifuged at 50000 x *g* for 180 min at 4°C. The final OM pellet was washed in distilled water and stored at -20°C.

2.11.2 Modified Booth and Curtis method. OMs were also prepared by the method of Booth and Curtis (1977). Cells from 500 ml cultures were washed twice in

0.85% saline and then resuspended in 10 ml 0.25M sucrose, 33 mM tris(hydroxymethyl) aminomethane buffer pH 8.0. Lysozyme and EDTA were added to final concentrations of 30 μg/ml and 250 μg/ml respectively and the suspension allowed to stand at room temperature for 10 min. The cells were then poured into four volumes of sterile distilled water at 4°C and stirred until mixing was complete. Cells were then broken by three passages through a French Pressure cell at 5 ton per square inch pressure. Remaining whole cells were removed by centrifugation at 3000 *g* for 20 min and the supernatant centrifuged at 50000 *g* for 180 min at 4°C to sediment cell membranes. The membrane pellet was washed three times in sterile distilled water at 4°C, resedimented and finally resuspended in 1 ml sterile distilled water.

2.11.3 DNase, RNase, lysozyme method. This method was based on that of Osborn and Wu (1980). The washed cell pellet from 500 ml of culture was resuspended in 5 ml of sterile distilled water and the cells broken by three passages through a French Pressure cell at 5 ton per square inch pressure. Lysozyme, RNase (type XII-A, Sigma), and DNase (type IV, Sigma) were then added to a final concentration of 30 μg/ml, 10 μg/ml and 10 μg/ml respectively. The suspension was then allowed to stand for 10 min at room temperature. Whole cells were removed by centrifugation at 3000 *g* for 5 min and the supernatant centrifuged at 50000 *g* for 180 min to sediment cell membrane material. The pellet was then washed four times by resuspension in 10 ml sterile distilled water and resedimentation at 50000 *g* for 10 min. The pellet was finally resuspended in 2.5 ml sterile distilled water.

2.11.4 Modified method using no enzymes. Washed cells from 500 ml of culture were suspended in 10 ml of water and broken by three passages through a French Pressure cell at 5 ton per square inch pressure. Whole cells were removed by centrifugation at 3000 *g* for 5 minutes and the supernatant then centrifuged at 50000 *g* for 180 min to sediment the cell envelope material. The resulting pellet was then washed four times by resuspending the pellet in sterile distilled water and resedimentation of the cell envelope material by centrifuging for 10 min at 50000 *g*. The pellet was finally resuspended in 2.5 ml sterile distilled water.

2.11.5 Separation of membranes by sucrose density gradient centrifugation. Discontinuous (or stepped) sucrose gradients were prepared in polyallomer tubes (14 x 89 mm, Beckman) using 1.6 ml each of aqueous sucrose solutions of the following % w/w concentrations in water: 65, 60, 55, 50, 45, 40, 35. The washed membrane samples (0.5 ml) were prepared by any of the methods described above mixed with 0.5 ml of 20% w/w aqueous sucrose, layered onto the step gradients and covered with 0.5 ml of water. The tubes were centrifuged for 20 h at 38000 rpm in a Beckman SW40 Ti rotor at 5°C. Fractions (0.25 ml) were collected in a microtitre plate after piercing the base of the tube with a syringe needle. Membrane bands (proteins) were detected by measuring the absorbance of each fraction at 340 nm (Anthos plate reader). β-Lactamase activity was assayed at 492 nm using a nitrocefin substrate. Samples from each fraction (0.02 ml) were diluted in 4 mM sodium phosphate buffer, pH 7.0 (0.130 ml) at 37°C in microtitre plate wells. Nitrocefin solution (0.1 ml, 0.05 mg/ml) was added to each well simultaneously with a multichannel pipetté and the change in absorbance monitored at 492 nm at 10 sec intervals over 5 min (Anthos plate reader).

2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of ammonium sulphate fractions and OM proteins was carried out by gel electrophoresis using either the Mini-Protean II system or large Protean slab gel apparatus (both Bio Rad). The Tris-HCl system described by Lugtenburg *et al.,* (1975) was used with 12 or 14% gels.

2.13 Visualisation of proteins in SDS-PAGE gels.

To visualise protein the gels were fixed and stained in 0.1% Coomassie Brilliant Blue (BDH) in 50% v/v methanol/ 10% v/v acetic acid and destained in 5% v/v methanol/ 10% v/v acetic acid. Molecular weights were estimated by comparison of Rf values with those of standard molecular weight marker proteins. The molecular weight markers used were: α-lactalbumin, 14.2 kD; soybean trypsin inhibitor, 20.1 kD; trypsinogen, 24 kD; carbonic anhydrase, 29 kD; glyceraldehyde-3-phosphate, 36 kD,

egg albumin, 45 kD; and bovine albumin, 66 kD.

2.14 Visualisation of β-lactamase activity in SDS-PAGE gels

2.14.1 Nitrocefin staining. The SDS-PAGE gel was removed from the glass plates and washed, to remove SDS and renature the enzyme, in 5mM potassium phosphate buffer (pH 7.0) on a reciprocating shaker for 180 min at room temperature with 10 changes of buffer (Tai *et al.*, 1985). The gel was covered with a dilute solution of nitrocefin (0.005 mg/ml) for 5 min, this was then decanted and the gel was flooded with a more concentrated solution of nitrocefin (0.05 mg/ml). The development of pink bands (produced by the hydrolysis of nitrocefin) indicated the presence of β-lactamase. The gels were then photographed using Kodacolor Gold film (ASA 400). The position of any bands was then marked by stabbing the gel with a needle dipped in Indian ink and the gel stained for protein with Coomassie Blue R-250.

2.15 Neuraminidase treatment of β-lactamase and sialic acid estimation

Neuraminidase (sialidase) releases N-acetyl neuraminic acid (sialic acid) from a wide variety of glycoproteins. Treatment of such complexes with neuraminidase should lead to the release of the native enzyme and sialic acid. Enzymic complexes that are sensitive to neuraminidase can exist in any bacterium regardless of the type of β-lactamase involved. The assay for sialic acid is based on an assay for neuraminidase activity which measures the amount of sialic acid released from bovine submaxillary mucin (Aminoff, 1961).

Crude cfe from *P. aeruginosa* clinical strain 258 was prepared by the method described in section 2.8.1 and diluted with 5 mM phosphate buffer to give a solution calculated as being equivalent to 1% (10 mg $ml^{-1)}$ protein. Aliquots of 400 µl of this solution were placed in each of two tubes and 500 µl of 0.1 M acetic acid was added to each. The tubes were then placed in a 37°C water bath. 100 ml of a solution of 1 mg/ml neuraminidase was added (equivalent activity = 1 mg = 8 units activity) to tube 1 and 100 ml of sterile distilled water added to tube 2, which constituted the control. Both tubes were then incubated for 3 h at 37°C. After 3 h 1 ml of 5% phosphotungstic acid was added to each tube.

The tubes were then centrifuged for 10 min. 500 µl of the supernatant was then transferred to a clean glass tube and 100 ml of 0.2 M sodium metaperiodate added. The reaction mixtures were then allowed to stand at room temperature for 20 min after which 1 ml of 0.755 M sodium arsenite was added. The tubes were then shaken until the brown colour disappeared and 3 ml of 0.6% thiobarbituric acid were added. The tubes were then heated in a boiling water bath for 15 min, followed by cooling on ice for 5 min and the addition of 4.6 ml of cyclohexanone. The tube contents were thoroughly mixed by vortexing. The tubes were then centrifuged at 13000 *g* for 15 min and the coloured cyclohexanone layer removed and the absorbance read at 549 nm. The sialic acid liberated by neuraminidase treatment could then be calculated from:

$$[A_{549} \text{ (sample)} - A_{549} \text{ (blank)}] \times 0.075$$

2.16 Peptide fragment patterns of inhibited β-lactamase

The purified β-lactamase (0.5 mg) prepared in section 2.5.8 was dissolved in 5 ml of 100 mM MOPS, 0.5 M NaCl, pH 7.0 containing 0.1 mg/ml tazobactam and incubated at 37°C for 6h. A control preparation containing the enzyme but no inhibitor was also prepared. The reaction mixtures were then dialysed against 2 x 2 l volumes of 1 mM MOPS, pH 7.0, containing 1 mM NaCl, for 48h at 4°C, to remove unbound tazobactam. The inactivated and control enzyme were then freeze-dried. The inactivated and control enzyme samples were then dissolved in 100 ml of 1% (w/v) NH_4HCO_3 and treated with 0.1 mg of trypsin (type XIII, 1-chloro-4-phenyl-3-tosylamino-butan-2-one treated, Sigma) dissolved in 1 mM HCl (20 ml), for 3h at 37°C. A trypsin blank containing no β-lactam was also prepared.

The peptide fragments were separated by HPLC on a C_{18} reverse phase silica column (Waters µBondapak™ C18) using a gradient elution system provided by Walters 600 Multisolvent Delivery System consisting of Fluid Unit and Fluid Controller (Waters Associates, Milfield, MA, 01757, USA) linked to a Cecil Digital UV spectrophotometer with variable wavelength, (Cecil Instruments, Cambridge, England) and a chart recorder (J.J Lloyd Instruments, Southampton, England) set at 5 mm/min and a sensitivity of 100mV. The peak elution profile was monitored at either 214 or 230 nm. The digested samples were dissolved in 60 µl of elution buffer A (50

mM NH_4HCO_3, pH 7.8) and injected onto the HPLC column using 50 µl loading loop. Gradient elution (1mg/min) was performed by controlled changes in the ratio of buffer A and B (20% v/v 50 mM NH_4HCO_3, pH 7.8 in acetonitrile). Peptide fragments were separated using a linear elution gradient from 1% to 60% B over 30 minutes and then increasing the concentration of B to 100% over the following 5 minutes and holding this concentration for 10 minutes. After a further 10 minutes at 100% B to wash the column, B was returned to 1% (i.e. 99%A) (over 1 minute) to prepare the system for the next injection. Eluted fragments showing absorbance at 230 nm were collected. Elution profiles produced from digests of inhibited β-lactamase were compared to uninhibited controls and in turn to blank injections in order to eliminate changes in gradient baseline. Fractions collected from the HPLC of the tryptic digest of the inhibited enzyme were evaporated to dryness and subjected to acid hydrolysis with 6M HCl (1 ml) for 4 h at 100°C hours in a Pyrex glass tube sealed under nitrogen. After hydrolysis the opened hydrolysis tube was placed in a vacuum dessicator in the presence of NaOH pellets (to absorb HCl) and concentrated sulphuric acid (to absorb water). When the sample in the tube had dried (18 h at room temperature) 1 ml of water was added and the procedure repeated twice more. This procedure effectively removed all residual HCl from the sample. Amino acid analysis of the acid hydrolysate was then carried out by Dr. J. Fox of Alta Bioscience, Department of Biochemistry, Birmingham University using ion exchange chromatography (LKB) with post-column ninhydrin derivatisation. The identity of each amino acid was then determined by comparison with authentic standards and estimated quantitatively by peak areas.

2.17 Crystallographic investigation of tazobactam

The free acid of tazobactam (YTR-830, 3-methyl-7-oxo-3-(1-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide) was crystallised from a 70:30 ethanol/water mixture as colourless stout hexagonal prisms. A specimen 0.83 mm long x 0.33 mm wide x 0.40 mm thick was selected for determination of unit cell parameters and collection of intensity density data using an Enraf-Nonius CAD4 diffractometer with graphite-monochromated Mo-Kα radiation, lambda = 0.71069 Å. The unit cell parameters were refined by least squares analysis

of setting angles of 25 reflections, $11.5 \leq \theta \leq 14.1°$. Intensity data were collected by the $\omega - 2\theta$ scan technique with ω scan range $(1.0 + 0.35 \tan \theta)$ and ω scan speed 4.0 to 4.0 deg min^{-1}.

The structure was solved by direct methods using the RANTAN procedure within MULTAN (Main *et al.*, 1980). After missing non-hydrogen atoms were located in an electron density map, least squares refinement was carried out with SHELX (Sheldrick, 1976). All hydrogen atoms except those in carboxyl groups were introduced in calculated positions determined by the molecular geometry, methyl groups were treated as rotatable rigid bodies, and other H atoms were assumed to ride on their attached atoms. Carboxyl hydrogen atom H(12) was located unambiguously in a difference electron density map. Coordinates and isotropic temperature factors were refined for H(12) and H(12') in the final cycles, along with isotropic temperature factors for other H atoms, coordinates and anisotropic thermal parameters for non-hydrogen atoms, and an empirical extinction correction. The observed reflections were weighted by $1/[\sigma 2(F) + 0.000519\ F2]$.

2.18 Computer studies

The GENBANK, EMBL, NBRF and SWISSPROT nucleic acid and protein sequence databases were searched using the University of Wisconsin Genetics Computer Group (UWCGC) program package. All searching was conducted via JANET on the SEQNET facility at the SERC Daresbury Laboratory, Warrington. Protein structures from the Brookhaven Protein Databank and small molecule atomic coordinates from the Chemical Databank System (Cambridge Structural Database) were also accessed at the Daresbury Laboratory. The following molecular modelling packages were employed: Chem-X (Chemical Design Ltd, Oxford), operating on the Aston University Vax 8650 mainframe processor with a Sigmex 5684 graphics terminal; QUANTA (version 2.0)/CHARMm (Polygen Corporation, Massachusetts), operating on a Silicon Graphics Iris 3120 workstation; CAChe/MOPAC (Stewart, 1989), operating on a Tektronix CAChe workstation The protein secondary structure prediction program, PROTYLSE (Scientific and Educational Software) was run on an

Amstrad 1640 personal computer.

2.18.1 Manual amino acid sequence alignment. The test sequence and master sequence were first aligned by matching up the conserved and active site residues. The remainder of the sequence was then aligned so that as many residues as possible were coincided with an identical or chemically similar residue by introducing gaps into the sequence where necessary

2.18.2 Active site modelling. Modelling and subsequent studies of the active site of the chromosomal β-lactamase of *P. aeruginosa* were carried out using the facilities available in the following molecular modelling packages: Chem-X, operating on the Aston University Vax 8650 mainframe processor with a Sigmex 5684 graphics terminal; QUANTA (version 2.0)/CHARMm (Polygen Corporation, Massachusetts), operating on a Silicon Graphics Iris 3120 workstation.

2.18.3 Electrostatic potential maps. The electrostatic isopotential surface of tazobactam (Purvis, 1991) was calculated, after MOPAC (Stewart, 1989) minimisation, using the CAChe workstation. The electrostatic isopotential surface was drawn over the MOPAC minimised structure. The +/-0.05 a.u. (+/-31.4 kcal mol^{-1} or +/-131.3 kJ/mol^{-1}) surfaces were coded blue for negative and red for positive electrostatic potential..

2.18.4 2-D structure prediction. The two dimensional structures of all the amino acid sequences under investigation were predicted using the 2-D structure prediction package PROTYLSE. This predicts the propensity of an amino acid sequence to adopt a particular conformation using the approaches developed by Garnier, Osgothorpe and Robson (GOR) (1978) or Chou and Fasman (1978a; 1978b; 1979).

RESULTS
CHAPTER 3

3. ISOLATION OF *P. AERUGINOSA* β-LACTAMASE

3.1 Aims

The aims of this section of work were:

(1) to isolate and purify the constitutive chromosomal β-lactamase from *P. aeruginosa* SAI^{const} and

(2) to determine its characteristics.

3.2 Antibiotic susceptibilities of *P. aeruginosa* SAI^{const}

P. aeruginosa SAI^{const} is a laboratory strain which is derepressed for expression of its chromosomal β-lactamase. The strain was shown, by IEF, to express only this chromosomal β-lactamase (pI range 7.2-7.4) and not to contain any plasmid mediated β-lactamases.

The strain was found to be resistant to a number of antibiotics. The MIC values of some antimicrobial compounds for *P. aeruginosa* SAI^{const} are shown in Table 5.

Table 5. Antibiotic susceptibility of *P. aeruginosa* SAI^{const}

Compound	MIC (μg/ml) for *P. aeruginosa* SAI^{const}
Cefotaxime	>128
Ampicillin	>512
Cloxacillin	256
Azlocillin	128
Carbenicillin	256
Cefoperazone	128
Chloramphenicol	128
Clavulanic acid	>512
Sulbactam	>512
Tazobactam	>512

P. aeruginosa SAI^{const} was resistant to many of the antipseudomonal penicillins and cephalosporins tested.

3.3 Isolation of *P. aeruginosa* SAI^{const} β-lactamase

The chromosomal β-lactamase produced by *P. aeruginosa* SAI^{const} was isolated and purified using the techniques of ammonium sulphate precipitation and gel filtration. A summary of the purification scheme is shown in Table 6.

3.3.1 Ammonium sulphate precipitation. The chromosomal β-lactamase produced by *P. aeruginosa* SAI^{const} was isolated using the technique of ammonium sulphate precipitation (see section 2.8.1). After stage 3 of the purification procedure the majority of the β-lactamase activity was present in the 70% ammonium sulphate precipitated fraction.

3.3.2 Gel Filtration. The dialysed 70% ammonium sulphate precipitate from stage 3 was purified by gel filtration FPLC. Figure 5 shows the results.

The results show that the β-lactamase activity and protein eluted in two major peaks; one corresponding to an approximate relative molecular weight of 43 kD (fractions 21-26) and a second at an approximate relative molecular weight of 58 kD (fractions 9-16), and one minor peak corresponding to an approximate relative molecular weight of 94 kD (fractions 3-7). Fractions 21-26 were pooled and used in subsequent kinetic assays.

3.4 Isoelectric focusing

IEF of the whole dialysed 70% ammonium sulphate precipitates from stage 3 of the purification of *P. aeruginosa* SAI^{const} β-lactamase and gel filtration fractions 5, 12, and 24 (samples corresponding to each peak of the gel filtration profile) of the dialysed 70% ammonium sulphate precipitates gave pI values of 7.2 for all samples. No difference in pI was observed between the different molecular weight β-lactamases.

3.5 Determination of molecular weight.

The relative molecular weight was estimated by gel filtration FPLC through Superose 12 HR 10/30 cross-linked agarose and by SDS-PAGE. From gel filtration FPLC the major chromosomal enzyme was estimated to have a relative molecular

weight of 45 kD and the higher weight β-lactamase components a relative molecular weight of 62 kD and 98 kD respectively. From SDS-PAGE the relative molecular weight of the major β-lactamase was found to be 42.5 kD, and 58 kD and 94 kD for the higher molecular weight β-lactamases.

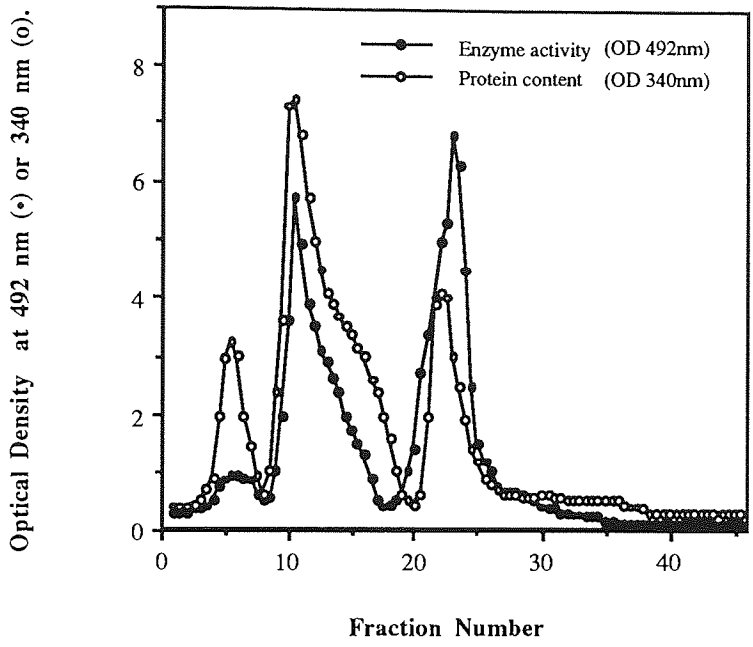


Figure 5. The β-lactamase activity and protein content profile of the FPLC gel filtration fractions of the dialysed 70% ammonium sulphate fraction. The dialysed 70% ammonium sulphate precipitate from stage 3 of the purification scheme was separated into its component proteins using gel filtration. The β-lactamase activity (measured as the absorbance of nitrocefin solution (100 µl) at 492 nm after addition of 20 µl of fraction) and protein content (absorbance of fraction at 340 nm) were measured for each gel filtration fraction and plotted against fraction number. Each fraction is equivalent to 0.1 ml of column eluate. The β-lactamase and proteins of the 70% ammonium sulphate precipitate elute as two major peaks (relative molecular weight 58 kD and 43 kD respectively) and one minor peak (94 kD).

3.6 Freeze drying

The fractions from stage 4 that showed the greatest β-lactamase activity (fractions 21-26) were pooled and freeze-dried. This process resulted in a white powdery solid which gave one major protein band at 42.5 kD, which corresponded to the position of β-lactamase activity in nitrocefin-stained gels, and several minor protein bands corresponding to relative molecular weights of 58 kD, 36 kD and 16 kD on SDS-PAGE.

3.7 Comparison of activities during purification

Table 6 shows a summary of the purification scheme used for the β-lactamase of *P. aeruginosa* SAI^{const}.

Table 6. Summary of the purification schedule for the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}

Stage	Procedure	SA *	TP †	TA #	Fold purification	Yield %
1	Ultrasonic disintegration	1960	740	$1.45x10^6$	1	100
2	Ammonium sulphate precipitation	80000	17.63	$1.41x10^6$	41	97
3	Ammonium sulphate precipitation	161000	7.63	$1.23x10^6$	82	84.8
4	Gel Filtration	313000	3.7	$1.15x10^6$	159	79.7
5	Freeze Drying	637000	1.56	$9.93x10^5$	325	68.4

* specific activity nmoles nitrocefin hydrolysed/min/mg protein
† total protein content mg
# total activity nmoles nitrocefin hydrolysed/min/mg protein

β-Lactamase was measured spectrophotometrically from the rate of nitrocefin hydrolysis and protein content was measured using the Lowry method. β-Lactamase activity of samples refers to total β-lactamase activity i.e. activity of major β-lactamase + activity of higher molecular weight forms. Specific enzyme activity of each fraction was calculated as the total units of β-lactamase protein (moles of substrate reacted per minute) / total amount (mg) of all proteins present. Total activity of each fraction was calculated as the specific activity of the fraction x the total mg of protein in the fraction. The yield expresses the recovery of the β-lactamase as the result of a particular fractionation step and was calculated as the total activity of a given fraction / the total activity of the original preparation. The fold purification expresses the purity of the fraction with respect to the β-lactamase relative to that of the original starting preparation and was calculated as the specific activity of the fraction / specific activity of the preparation.

The scheme gave a gradual increase in the specific activity of the fractions (as would be expected), from 1960 nmoles nitrocefin hydrolysed/min/mg protein in the broken cell homogenate to 637000 nmoles nitrocefin hydrolysed/min/mg protein in the final freeze dried preparation. The final yield of purified enzyme was 68.4%, for this preparation.

3.8 Isolation of high molecular weight β-lactamases

On SDS-PAGE of the cfes from *P. aeruginosa* strains 10, 71, 75, 177, 241, 258, PAO1DR and SAI^{const}, (Figure 6), one major band of β-lactamase was observed at 42.5 kD, however, minor bands of high molecular weight β-lactamase activity were observed in the cfes of strains 10, 75, 177, 241, 258 and SAI^{const}. Three bands of β-lactamase activity were observed in the cfes of strains 10, 241 and 258; these corresponded to relative molecular weights of 42.5, 58 and 94 kD. Two bands of β-lactamase activity were observed in the cfes of strains 75, 177 and SAI^{const}; these corresponded to relative molecular weights of 42.5 and 58 kD. (Only a single band of β-lactamase activity was observed in the cfes of strains 71 and PAO1DR, corresponding to a relative molecular weight of 42.5 kD).

The additional β-lactamase bands may be due to the aggregation of enzyme molecules. Possibly the 94 kD enzyme could be due to this phenomenon, but this does not explain the presence of the 58 kD band; also any aggregate should decompose on SDS-PAGE.

3.9 Investigation of high molecular weight β-lactamases

The cfes from *P. aeruginosa* strains 10, 75, 177, 241, 258 and SAI^{const}, which all exhibited additional high molecular weight protein bands with β-lactamase activity, were incubated with and without neuraminidase as described in section 2.15 to investigate the possibility of the enzyme being linked to sialic acid.

Samples of the cfe were incubated with neuraminidase or with sterile distilled water (without neuraminidase sample) for 2h at 37°C. Control cfe samples were incubated with sterile distilled water on ice to assess the degree of non-specific protease action which might cause degradation of the sample and/ or enzyme complexes. The treated and untreated cfes were run on SDS-PAGE gels and β-lactamase activity visualised as described in section 2.14.1. The results are shown in Figure 7.

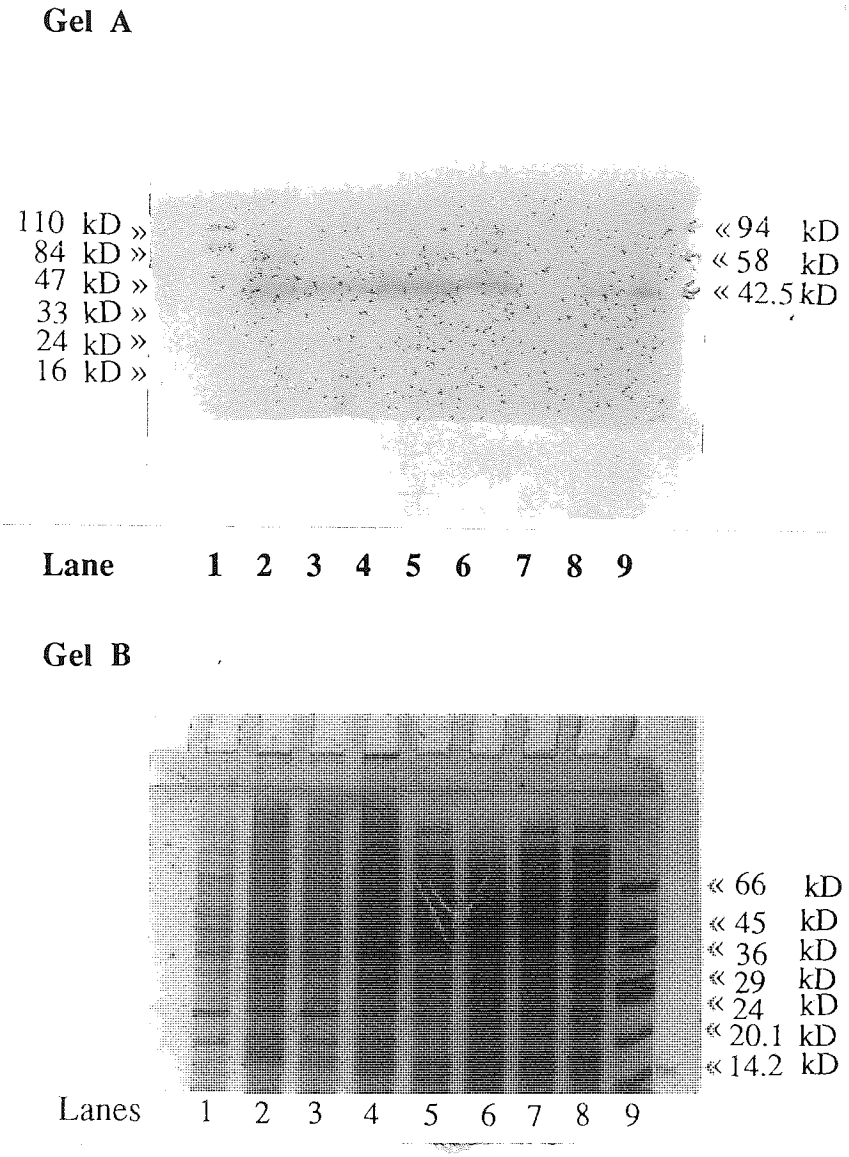


Figure 6. SDS-PAGE of cfes from *P. aeruginosa* strains 10, 71, 75, 177, 241, 258, PAO1DR and SAI^{const}. The cfes from *P. aeruginosa* strains 10, 71, 75, 177, 241, 258, PAO1DR and SAI^{const} were separated by SDS-PAGE. Gel A shows the cfes from *P. aeruginosa* strains 10 (lane 2), 71 (lane 3), 75 (lane 4), 177 (lane 5), 241 (lane 6), 258 (lane 7), PAO1DR (lane 8) and SAI^{const} (lane 9) and pre-stained molecular weight markers (lane 1) stained with nitrocefin to visualise β-lactamase activity. Gel B shows the cfes from *P. aeruginosa* strains 10 (lane 1), 71 (lane 2), 75 (lane 3), 177 (lane 4), 241 (lane 5), 258 (lane 6), PAO1DR (lane 7) and SAI^{const} (lane 8) and molecular weight markers (lane 9) stained with Coomassie blue to visualise protein. Three bands of β-lactamase activity were observed, the lower stronger band corresponded to a relative molecular weight of 43 kD whilst the upper bands corresponded to relative molecular weights of 58 kD and 94 kD.

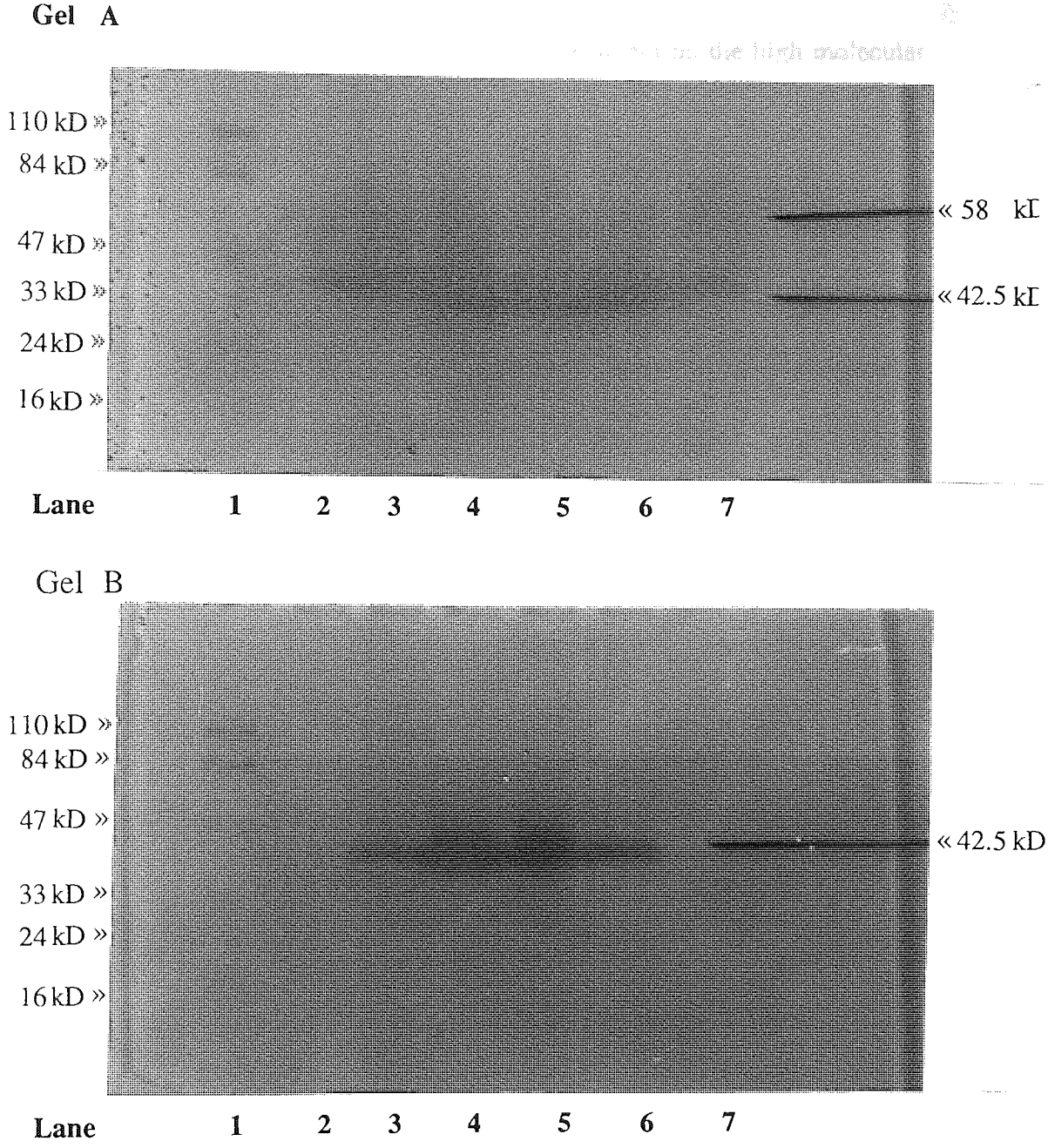


Figure 7. Effect of neuraminidase treatment on high molecular weight β-lactamase activity. Cfes from *P. aeruginosa* strains 75, 177, 241, 258 and SAI^{const} were incubated with and without neuraminidase and then separated using SDS-PAGE. Gel A shows the cfes from *P. aeruginosa* strains 75, 177, 241, 258 and SAI^{const} incubated without neuraminidase for 2 h at 37°C (lanes 2-7) and pre-stained molecular weight markers (lane 1) stained with nitrocefin to visualise β-lactamase activity. Gel B shows the cfes from *P. aeruginosa* strains 75, 177, 241, 258 and SAI^{const} incubated with neuraminidase for 2 h at 37°C (lanes 2-7) and pre-stained molecular weight markers (lane 1) stained with nitrocefin to visualise β-lactamase activity. Two bands of β-lactamase activity were observed in the untreated samples, the lower stronger band corresponded to a relative molecular weight of 42.5 kD whilst the upper band corresponded to a relative molecular weight of 58 kD. Only a single band of β-lactamase activity was observed in the neuraminidase treated samples, corresponding to a relative molecular weight of 42.5 kD.

Figure 7 shows the effects of neuraminidase treatment on the high molecular weight β-lactamase activity. Gel A shows the cfes from *P. aeruginosa* strains 75, 177, 241, 258 and SAI^{const} which all demonstrate multiple weight β-lactamases. The samples have all been incubated with sterile distilled water at 37°C for 2 h. Two bands of β-lactamase activity were observed corresponding to relative molecular weights of 42.5 kD and 58 kD. Gel B shows the cfes from *P. aeruginosa* strains 75, 177, 241, 258 and SAI^{const} after incubation with neuraminidase at 37°C for 2h. Only a single band of β-lactamase activity was observed corresponding to relative molecular weight of 42.5 kD. No upper band was present which suggests that the high molecular weight β-lactamase complex had been destroyed by the action of the neuraminidase.

3.10 Estimation of the amount of sialic acid bound to β-lactamase in strain 258.

Assay of the sialic acid released from the β-lactamase of strain 258 showed that each mole of β-lactamase was associated with 0.04 moles of sialic acid. The sialylated form appears to be only a minor form of the enzyme (approximately 2%) in strain 258.

3.11 Discussion.

The results of the purification procedure used for the chromosomal β-lactamase from *P. aeruginosa* SAI^{const} are summarised in Table 6. Like most chromosomal β-lactamases from Gram-negative organisms the enzyme hydrolysed cephalosporins more rapidly then penicillins and so was a cephalosporinase. The properties of the enzyme corresponded to those of a typical cephalosporinase, described by Sawai *et al.* (1973), which corresponds to Class I β-lactamases as described by Richmond and Sykes (1973). The enzyme was purified about 325 fold from the crude extract and gave a single major protein band on SDS-PAGE gel electrophoresis. The enzyme could be blotted on to nitrocellulose and still remained active.

The approximate molecular weight of the chromosomal β-lactamase was estimated to be 42.5 kD by both gel electrophoresis and gel filtration. Additional higher weight forms of the β-lactamase enzyme could be detected in some but not all of the

samples. These higher molecular weight forms corresponded to relative molecular weights of 58 and 94 kD. SDS-PAGE of samples from the three peaks obtained on gel filtration, that showed β-lactamase activity and suggested three different molecular weights of 43, 58 and 94 kD, gave only a single molecular weight band of 42.5 kD.

The existence of high molecular weight forms of β-lactamase bound to sialic acid-containing structures has been observed with TEM from *E. coli* R6K, PSE-1 from *P. aeruginosa* Pu21 (RPL11), and PSE-1 from *P. aeruginosa* wild strains (Jouvenot *et al.*, 1984). The isoelectric focusing of β-lactamases have produced complicated patterns, with a main band, whose pH was taken as the isoelectric point (pI) and satellite bands. A number of suggestions have been put forward to explain this phenomenon including differences in amino acid content (Simpson and Plested, 1983) which result in differences in pI, or for single enzymes, isoenzymes may result in satellite bands on IEF. However, the complicated focusing patterns of β-lactamases has never been fully explained. An alternative explanation suggested for the microheterogenity in isoelectric focusing of many proteins could be differences in the sialic acid content of the various bands. Sialic acid linked proteins would have a more acid pI than the native protein.

The results obtained from the IEF of *P. aeruginosa* extracts were not sufficiently clear to determine the presence or absence of satellite bands or distinguish the high molecular weight form from the normal molecular weight form. However, initial experiments with neuraminidase treatment suggested that it was the presence of sialic acid in the high molecular weight β-lactamases which caused the weight difference. The high molecular weight β-lactamase could be removed by neuraminidase treatment. Incubation of the samples without neuraminidase at 37°C does not result in loss of the high molecular weight form of the enzyme. The high molecular weight β-lactamase appears to be due to a specific interaction between β-lactamase and sialic acid. Dissociation of the complex-leading to release of the enzyme can only be effected by treatment with neuraminidase- an enzyme specific for sialic acid.

Sialic acid is a cell wall component, which suggests that a sialyated form of the enzyme may be associated with the cell wall or OM. The sialyated form of the enzyme could have some specific physiological role or it could contribute to resistance of the cells to β-lactam antibiotics. β-Lactams entering the cell must first cross the OM before

they reach their penicillin-binding targets. If β-lactamase was situated in the OM or cell wall then this would hydrolyse any invading antibiotic molecules before they did any harm to the cell. Any escaping β-lactams would then be subject to hydrolysis by the periplasmic enzyme. This could account for the high level of resistance of *P. aeruginosa* to a large number of β-lactam agents and could explain why the traditional methods of measuring cell permeability do not appear to work with this organism - especially those that are based on the diffusion of β-lactam compounds (Nichols, 1987; Livermore and Davy, 1991).

CHAPTER 4

4. EFFECT OF GROWTH CONDITIONS ON β-LACTAMASE EXPRESSION

4.1 Aims

The aims of this section of work were:

(1) to determine whether medium type, iron and penicillin G had effects on the production of β-lactamase and

(2) to determine whether medium type, iron and penicillin G had effects on the location of β-lactamase

4.2 Influence of growth environment on the production and distribution of β-lactamase.

P. aeruginosa strains 6750, 4082, 50DR, SAI^{const}, PAO1, PAO1DR, 3, 10, 31, 54, 71, 75, 113, 177, 236, 241 and 258 were grown in each of six different media (viz NB, NB+PenG, CDM-Fe, CDM+Fe, CDM-Fe+PenG and CDM+Fe+PenG) in batch culture. The cells were harvested and the β-lactamase extracts prepared as described in section 2.9 and 2.11 and assayed for protein content and β-lactamase activity. The results are shown in Table 7.

The first feature to note is the range of β-lactamase activities produced by growth in nutrient broth. PAO1, the control (non-derepressed) strain produced the lowest level of β-lactamase and clinical isolate 258 produced the highest level of β-lactamase. The clinical strains (selected as producing above average amounts of the enzyme compared to the control strain PAO1) exhibited a wide range of activities, from 18.6 nmoles nitrocefin hydrolysed/min/g cells (just above the level produced by the control strain) to 8490 nmoles nitrocefin hydrolysed/min/g cells for strain 258. Addition of penicillin G at a non-inhibitory level (500 μg/ml) significantly enhanced enzyme specific activity in all strains. In one clinical isolate (258) the induced level of β-lactamase in NB+PenG exceeded that of the highest β-lactamase-producing laboratory strain (50DR) grown in the same medium. The pattern of induction by penicillin G was consistent among the

clinical strains. Interestingly, PAO1DR, a derepressed strain, could be induced further by penicillin G.

Table 7. Activities of β-lactamase in clinical and laboratory strains of *P. aeruginosa* grown under various nutritional conditions.

Strain	Growth Conditions											
	NB		NB		CDM-Fe +Pen G		CDM+Fe		CDM-Fe +Pen G		CDM+Fe +Pen G	
	Hom	TM	Hom	TM	Hom	TM	Hom	TM	Hom	TM	Hom	TM
	nmoles of nitrocefin hydrolysed per min per g wet weight of cells											
3	4170	17.4	14600	285	41700	216	5020	1110				
10	192	24.6	4510	60	3330	74.3	77.1	1.8	47700	5200	7010	87
31	114	9.7	1310	305	5440	1030	314	109				
54	198	14.5	1900	18	6810	29	6720	3.6				
71	466	1.1	947	44	9950	1540	231	0.8	4390	508	2010	8.5
75	1035	4.7	2750	76	42300	142	1600	39.7	16900	6500	1390	1.4
113	152	0.9	718	276	705	98	286	142				
177	61.8	1	1000	504	3640	723	491	18	141000	20900	71600	1650
236	307	6.7	5010	2980	2650	184	95.3	8.8				
241	416	9.3	7480	905	8010	317	2620	0.6	6000	1800	4760	48.4
258	8490	495	30400	539	45100	57	4090	117	249000	3290	44700	448
PAO1	18.6	3	43	15	595	64.3	129	11.3	11200	69703	470	66
PAO1DR	8450	23.7	31900	2060	38700	492	5180	145	137000	1810	74900	1440
SAI^{const}	4940	43.1	19200	134	34800	10.7	9290	2.3	225000	2970	77100	14000
4082	130	1.3	6020	40	5070	120	42.9	12.9				
6750	38	1.6	195	30	316	40	64.3	0.3	15300	101	6320	66.2
50DR	10900	500	28900	1010	44200	1840	9100	304				

β–Lactamase activities of homogenised cells (Hom) and total membranes (TM) were measured spectrophotometrically from the rate of nitrocefin hydrolysis and protein contents measured using the Lowry method. Activities are expressed as nmoles of nitrocefin hydrolysed per min per g wet weight of cells. *P. aeruginosa* strains 3, 10, 31, 54, 71, 75, 113, 177, 236, 241, 258, PAO1, PAO1DR, SAI^{const}, 4082, 6750 and 50DR were grown in NB, NB+PenG, CDM-Fe, CDM+Fe, CDM-Fe+PenG and CDM+Fe+PenG in batch culture. Cells were harvested and the used culture medium retained; β-lactamase extracts prepared (Hom) and fractionated into cfe and TM. β-Lactamase activity was calculated as total activity of the fraction i.e. the specific activity of the fraction x total mg of protein in the fraction.

The same general pattern of enzyme specific activity was seen when the strains were grown in CDM+Fe. However, in CDM-Fe all of the strains showed a marked increase in specific activity. The influence of iron restriction was considerable, ranging from a five-fold increase in the specific activity of the cell free extracts from the non-derepressed laboratory strains, PAO1 and 6750, to a ten to twenty-fold increase in the clinical isolates. It would appear that the availability of iron has a regulatory effect on the expression of β-lactamase. In some strains the addition of penicillin G (500 μg/ml) to CDM (-Fe and +Fe) resulted in a further increase in β-lactamase activity, ranging from ten to fifteen-fold for the non-derepressed laboratory strains and five to forty-fold for the partially derepressed clinical strains. This suggests that the influence of iron on β-lactamase expression is distinct from the inductive effect of penicillin G.

The whole cell homogenate from each strain was fractionated by differential centrifugation into component fractions. The majority of the enzyme was present in the cell-free extract, representing periplasmic, and possibly intracellular enzyme. A small amount was also detected in the total membrane fraction. This material was initially thought to represent enzyme transiently located in the cytoplasmic membrane during translocation to the periplasm. However, when whole cell sonicates were treated with sarkosyl to solubilise the cytoplasmic membrane, some activity remained with the insoluble OM pellet. This implied that some of the β-lactamase was located in the OM. This phenomenon was investigated further, as described in chapter 5.

4.3 Discussion

The production of β-lactamase by these organisms may have a practical significance in helping to establish and maintain infections of the lung. The *P. aeruginosa* cells are protected from β-lactam therapy by a combination of these enzymes and slow growth rate. In the case of the mucoid form, the organism traps a layer of the β-lactamase around itself (Giwercman *et al.*, 1991). This localised high concentration of β-lactamase, which the organism appears to manufacture almost from the start of growth in the lung, protects it from antibiotic action and also gives some protection to other normally antibiotic sensitive organisms, if present, such as *H. influenzae*, which in turn aids the establishment of infection within the lung. High level expression of β-

lactamase in the lung is not just a phenotypic phenomenon since stable, partially-derepressed strains from patients in which β-lactam therapy was ineffective have been reported (Giwercman *et al.*, 1990). These strains naturally express high levels of β-lactamase which is elevated further by iron restriction.

Seemingly the environment of the CF lung exerts several important selective pressures on the invading pathogen. Firstly, the iron depleted environment slows the growth rate; this exerts a selectional pressure because slower growing cells are less sensitive to β-lactams hence their survival in the lung is promoted. Secondly, the presence of therapeutic β-lactams favours the selection and proliferation of β-lactamase hyper-producing mutants; hyper-production of β-lactamase protects the cells from the action of β-lactam antibiotics, allowing the mutants to persist and proliferate whilst sensitive strains perish.

In all of the clinical strains β-lactamase expression increased under iron depletion in CDM to levels significantly higher than those achieved in either iron replete CDM, NB or NB+PenG. β-Lactamase expression could be induced even further by the addition of penicillin G. Approximately half the total β-lactamase activity was present in the periplasm and half in the culture medium. Therefore, in some clinical strains, a combination of iron depleted conditions in the lung (Brown *et al.*, 1984) and therapy with β-lactam antibiotics increase β-lactamase expression to levels that could influence the effectiveness of therapy.

The mechanism of regulation by iron is not clear and has not been described in other Gram-negative bacteria. Since levels of β-lactamase can be induced further by penicillin G, the regulation appears to be distinct from the *amp*R system of enterobacteriaceae (Tuomanen *et al.*, 1991). Identification of a regulatory "iron box" upstream of the *amp*C gene of *P. aeruginosa* awaits further sequence analysis. Interestingly, polyclonal antiserum against *E. coli* Fur protein has been used to the *fur* gene in *P. aeruginosa* (Prince *et al.*, 1991). The amino-terminus of the amino acid sequence of the purified protein shows strong homology with the amino-terminus of the *E. coli* protein suggesting that *P. aeruginosa* might possess a Fur-like regulatory system. Possibly, the reported "non-specific induction" of β-lactamase in body fluids (Cullmann *et al.*, 1984) is a result of iron restriction imposed by transferrin or lactoferrin. The observation of a ferritin iron storage protein in *P. aeruginosa*

(Cheeseman *et al.*, 1990) further complicates the mechanism or iron regulation since this component would influence the level of intracellular iron in the organism.

CHAPTER 5

5. ASSOCIATION OF β-LACTAMASE WITH THE OUTER MEMBRANE

5.1 Aims.

The aims of this section were:

(1) to establish whether β-lactamase was present in the OM of *P. aeruginosa* strains.

(2) to determine whether the association of β-lactamase with the OM was a genuine association or just an artefact of preparation

(3) to determine whether this association was affected by growth conditions

(4) to determine whether the association of β-lactamase with the OM was a transient stage in the secretion of the enzyme.

5.2 Association of β-lactamase with OMs

P. aeruginosa SAI^{const} was grown in NB and its membranes extracted by either the sarkosyl method (OMPs) or by French pressing (TMPs) with or without DNase, RNase or lysozyme as described in section 2.11. The OM preparations were then separated by SDS-PAGE (section 2.12) and the gels treated to visualise β-lactamase activity and subsequently stained for protein as described in sections (2.13 and 2.14.1). The OM gels of *P. aeruginosa* SAI^{const} prepared by all three methods are shown in Figure 8, lanes 2-4.

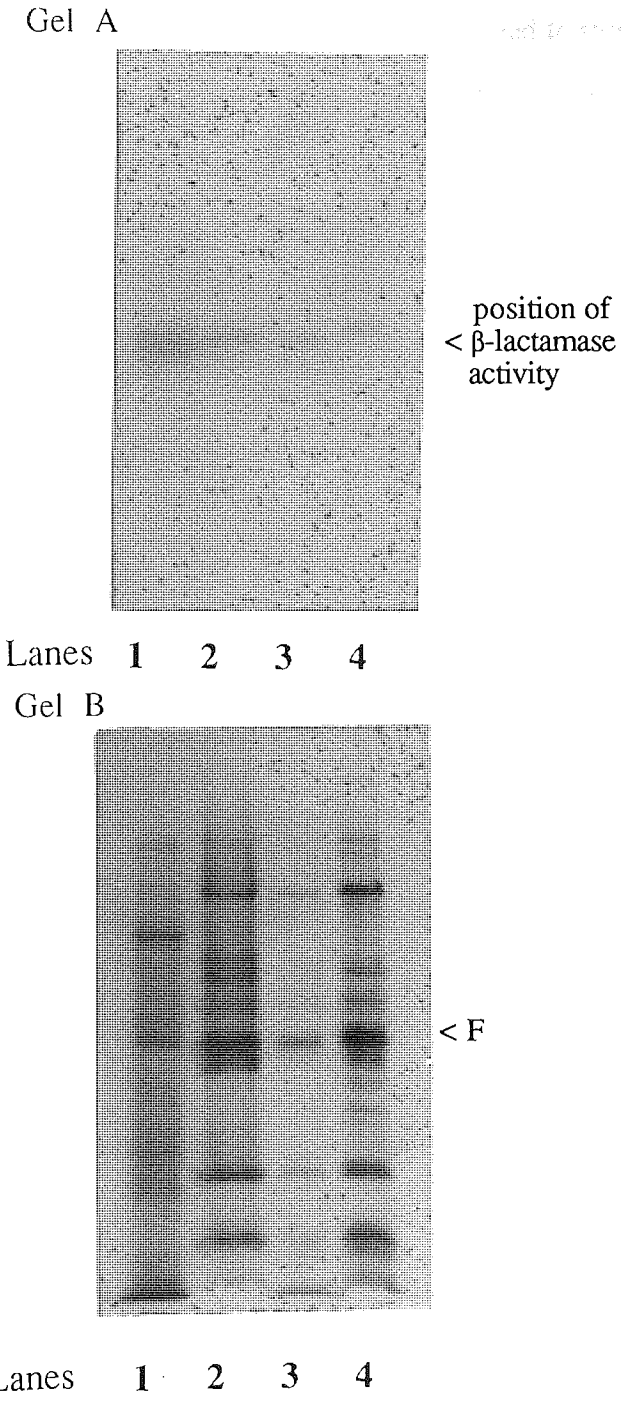


Figure 8. Association of β-lactamase with the outer membrane of *P. aeruginosa* SAI^{const}. The OMPs of *P. aeruginosa* SAI^{const} were extracted by the sarkosyl method (lane 2), French pressing with (lane 3) and French pressing without (lane 4) RNase, DNase and lysozyme. OMPs were separated using SDS-PAGE. The cfe of *P. aeruginosa* SAI^{const} was included as a control (lane 1) to show the position of the free enzyme. Gel A was stained with nitrocefin to visualise β-lactamase activity and subsequently stained with coomassie blue to visualise protein (Gel B). A single band of β-lactamase activity appeared running just above protein F. The protein profile shows the characteristic OM pattern although proteins D_1 and D_2 and H_1 and H_2 have not been separated.

Gel A was stained to visualise β-lactamase activity and subsequently stained to visualise protein (Gel B). The cfe of *P. aeruginosa* SAI^{const} was included to show the position of the free enzyme. Although some protein was lost during the β-lactamase staining process the gel shows that all the main OMPs (Protein E, F and G although Proteins D_1 and D_2, H_1 and H_2 have not been separated and appear as single bands) were present in *P. aeruginosa* SAI^{const}. There was very little variation in the OMP profile between the three methods of preparation. β-Lactamase activity was found in each sample corresponding to a relative molecular weight of 42.5 kD which ran just above Protein F on the gel. It would appear that β-lactamase is associated with the OM of *P. aeruginosa* strains.

5.3 Nature of β-lactamase association with OM

A series of experiments was performed to determine whether the association of β-lactamase with OM material was a genuine observation or whether it was an artefact of the preparative technique. The association may have been due to:

(1) an electrostatic interaction caused by the interaction of OM material and the detergent sarkosyl used in certain outer membrane preparations

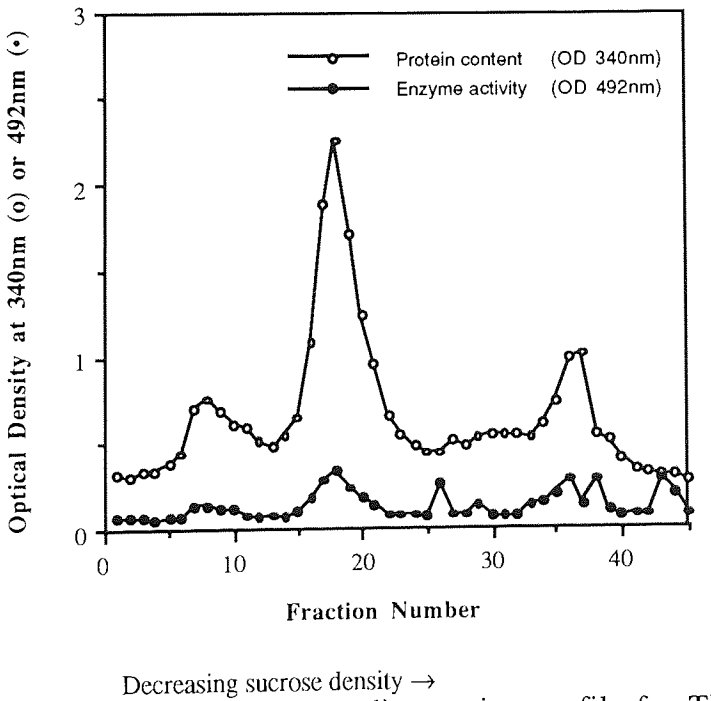
(2) trapping of the β-lactamase in OM micelles formed during the process of French pressing

OM preparations of *P. aeruginosa* PAO1 and *P. aeruginosa* SAI^{const} were made by French pressing without DNase, RNase or lysozyme (the modified enzyme method- a non-detergent method which eliminated sarkosyl from the preparations). Two additional preparations of the OMs of *P. aeruginosa* PAO1 were made by this technique, however, semi-purified β-lactamase was added to one before French pressing whilst semi-purified β-lactamase was added to the other after French pressing (this would eliminate the formation of enzyme micelles). The preparations were then processed according to section 2.10.4 before being separated by sucrose density gradient centrifugation and fractionated (150 μl fractions). The protein content and β-lactamase activity of each fraction was determined (2.9.2 and 2.10.2).

The protein content (absorbance at 340 nm) and enzyme content (absorbance of hydrolysed nitrocefin at 492 nm) was plotted against fraction number to give the

sedimentation pattern for each preparation.

The protein and enzyme sedimentation profiles of the three *P. aeruginosa* PAO1 preparations (*P. aeruginosa* PAO1 without enzyme, enzyme added before breakage, and enzyme added after breakage) showed three major protein peaks. No enzyme activity was measured in any of the OM preparations. This suggested that the enzyme detected in the OM preparations in section 5.2 was not due to the free β-lactamase (released periplasmic enzyme) being trapped in OM vesicles formed during French pressing or due to an electrostatic interaction caused by French pressing or the presence of sarkosyl in the OM preparations.



Decreasing sucrose density →

Figure 9. Protein and enzyme sedimentation profile for TMP of *P. aeruginosa* SAI^{const}. TMPs were prepared by the modified Booth and Curtis method and separated by sucrose density gradient centrifugation. The sucrose gradient was fractionated and the protein (measured as absorbance at 340 nm (o)) and enzyme (absorbance of hydrolysed nitrocefin solution at 492 nm (•)) content of each fraction measured. The absorbance values were plotted against fraction number.

The protein and enzyme sedimentation profile of *P. aeruginosa* SAI^{const} TMPs (Figure 9) showed three major protein peaks- fractions 4-12, 16-22 and 25-32 with which the enzyme activity was also associated.

These fractions obtained from the sucrose density gradient were then run on SDS-PAGE (results not shown) and found to contain mostly OM material. Fractions 4-12 contained predominantly Proteins D_1, D_2, E and F, fractions 16-22 contained all the major OM proteins whilst fractions 25-32 contained predominantly Proteins F, G and some H_1 and H_2. β-Lactamase activity was visualised as a single protein band running just above Protein F. The majority of enzyme activity was associated with fractions 4-12 and 16-22.

5.4. Effect of growth conditions on the association of β-lactamase with the OM.

P. aeruginosa strains 177, 241, PAO1DR, 4082, 71, 75, 50DR, 258, 6750, PAO1, 10 and SAI^{const} were grown in each of the four different media (i.e. NB, NB+PenG, CDM-Fe and CDM+Fe) and their OMPs extracted by the sarkosyl method. The OMP preparations were then separated by SDS-PAGE and the gels stained for β-lactamase activity followed by protein (results not shown).

β-Lactamase activity was found in OM preparations of: *P. aeruginosa* PAO1DR grown in NB and CDM-Fe; strain 71 grown in NB+PenG, CDM-Fe and CDM+Fe; strain 75 grown in NB and CDM-Fe; strain 177 grown in CDM-Fe and in strain 50DR grown in NB β-Lactamase activity was associated with a protein band running just above Protein F.

5.5 Effect of growth conditions on the protein and enzyme sedimentation profiles of the TMPs of *P. aeruginosa.*

P. aeruginosa strains 177, 241, PAO1DR, 4082, 71, 75, 50DR, 258, 6750, PAO1, SAI^{const} and 10 were grown in each of four media (NB, NB+PenG, CDM-Fe and CDM+Fe) and their TMPs prepared by French pressing without DNase, RNase and lysozyme. TMP preparations were separated by sucrose density gradient centrifugation and the gradients fractionated.

The protein and enzyme content of each fraction collected was determined by measuring the absorbance of the fraction at 340 nm (for protein) and 492 nm (hydrolysed nitrocefin solution for enzyme activity). The absorbance values were plotted against fraction number for each strain grown in each medium. The results

shown in Figure 9 were representative of the general trend shown by TMP preparations of all 10 strains when grown in NB, NB+PenG, CDM-Fe and CDM+Fe.

All of the protein profiles showed three major peaks. The protein profile of NB grown cells showed an additional peak. The highest protein concentration was found in the second peak with the exception of CDM-Fe grown cells where there was an increase in the heavy membrane material which may have been a result of the presence of high molecular weight IRMPs induced by the iron-deplete conditions. The proteins in the NB+PenG grown cells were not as well resolved and the profile showed an increase in the medium and light material which could have been due to the penicillin included in the medium causing damage to the cell wall.

The enzyme activity profile showed a similar pattern to the protein content profile. There were three major peaks of enzyme activity which coincided with the protein peaks with the exception of NB+PenG grown cells. In the NB+PenG grown cells the activity seemed evenly distributed throughout the OM profile.

5.6 Identification of protein material with which β-lactamase activity is associated

A TMP preparation of *P. aeruginosa* SAI^{const} was made using the modified Booth and Curtis method (2.11.2) and then separated by sucrose density gradient centrifugation. The fractions from the sucrose density gradient were then collected and the protein content and β-lactamase activity determined.

The protein sedimentation profile showed three major peaks of light, medium and heavy membrane material (Figure 9). The β-lactamase activity sedimentation profile showed that the enzyme activity was associated with the heavy peak. Very little enzyme activity was found in the subsequent fractions (medium and light membrane material)

The proteins isolated in the earlier fractions 15-24 were very characteristic of the heavy OM proteins. The lighter CM material was found in the later fractions and was distinct from the OM material. β-Lactamase activity was found only in fractions 5-15 which contained OM proteins, characterised by the presence of Protein F (41 kD) running just above this protein at 42.5 kD.

The fractions constituting each of the major peaks were pooled (fractions 7-11 =

Sample A, fractions 16-22 = Sample B, fractions 27-29 = Sample C and fractions 33-38 = Sample D) and assayed for protein content and β-lactamase activity. The total enzyme activity of each sample was calculated and compared to the total activity of the original whole cell homogenate.

The greatest β-lactamase activity was in sample B (12.31 nM/nitrocefin hydrolysed/min/ml) with a lesser amount in sample A (4.36 nM/nitrocefin hydrolysed/min/ml). Negligible activity was found in samples C and D (Table 8)

Table 8. Total β-lactamase activity and the percentage total β-lactamase activity in each of the four membrane samples and the cfe of *P. aeruginosa* SAI^{const}

Sample	total activity*	% total activity*
A	4.36	0.22
B	12.31	0.62
C	0.25	0.01
D	0.59	0.03
cfe	1971	99.0

* nmoles nitrocefin hydrolysed/min/ml

Fractions corresponding to each of the major protein peaks identified from the enzyme and protein sedimentation profile (Figure 9) were pooled (fractions 7-11= Sample A, fractions 16-22= Sample B, fractions 27-29= Sample C and fractions 33-38=Sample D) and their volumes adjusted to 1.5 ml. The protein content and β-lactamase activity of each sample were assayed (as described in sections 2.9.1 and 2.10.1). The total enzyme activity of each sample was calculated and compared to the total activity of the original whole cell homogenate.

In this preparation of *P. aeruginosa* SAI^{const} the total membrane associated β-lactamase activity constituted approximately 1% of the total β-lactamase activity of the cell.

5.7 Excretion of β-lactamase

To determine whether the excretion of β-lactamase was possible the propensity of the β-lactamase sequence to form an amphipathic α-helix was investigated using several

secondary structure prediction methods. The secondary structure prediction routine of Chou and Fasman (1979) was applied to the amino acid sequence of the *P. aeruginosa* AmpC β-lactamase (Lodge *et al.*, 1990) using the PROTYLZE Structure Predictor Software package to determine whether the chromosomal β-lactamase sequence had any of the properties of an excreted protein.

The secondary structure predictions (section 2.18.4) for the amino acid sequence of the mature chromosomal β-lactamase showed that there was a strong propensity for the 18 amino acid residues (residues 26-43, AGEAPADRLKALVDAAEQ, Table 16) on the amino-terminus to form an α-helix (after removal of the peptide leader sequence).

The Eisenberg method of amphipathic helix prediction was applied to the first 20 amino acid residues of the mature β-lactamase amino-terminus. The residues show a strong propensity for forming an α-helix which has pronounced hydrophobic and hydrophilic faces (see Figure 10).

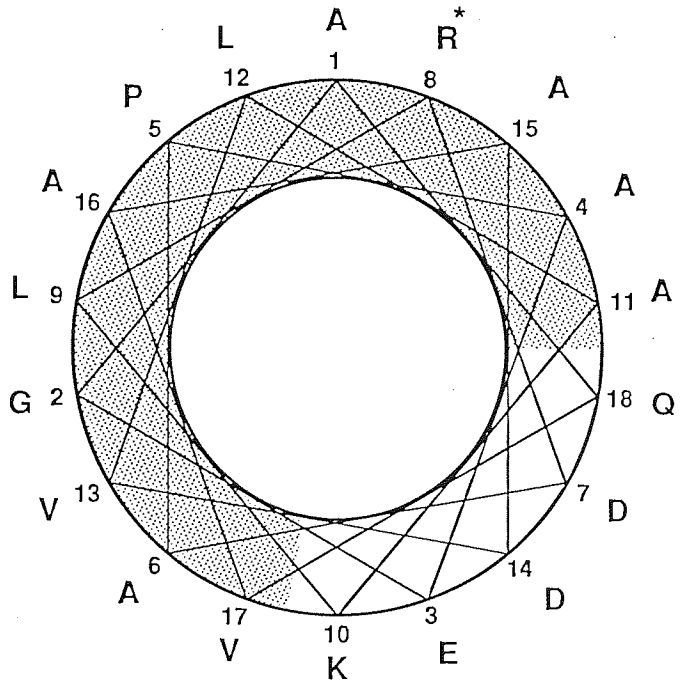


Figure 10. Helical wheel prediction of amphipathicity for the β-lactamase of *P. aeruginosa* SAI^{const}. The hydrophobic moment of the first 18 amino acids (residues 26-43 in Table 16) in *P. aeruginosa* SAI^{const} β-lactamase was calculated using the Eisenberg method and displayed on a helical wheel. Amino acids are represented as the one letter code and are numbered from 1 to 18. The shaded region shows the hydrophobic area of the helix and the clear region shows the hydrophilic area of the helix. Arginine (R*) is the only anomaly - a hydrophilic amino acid occurring in the hydrophobic region.

The calculated helical wheel projection of the amino terminal segment of the mature β-lactamase is strongly in favour of amphipathic helix formation. Two well-defined hydrophobic and hydrophilic regions are shown by the α-helix.

5.8 Discussion.

Resistance of *P. aeruginosa* strains to β-lactam antibiotics is due to a combination of several factors. A reduction in the level of permeability of the OM to β-lactam antibiotics may reduce the number of antibiotic molecules penetrating the cell as well as exclude certain antibiotic molecules due to their structural and chemical characteristics; the production of β-lactamase enzymes may trap or hydrolyse invading β-lactam molecules preventing them from reaching their sensitive PBP targets; and alterations in the PBP targets themselves may mean that the β-lactam can no longer bind. The results of this chapter suggest another location which could be important to β-lactam resistance is the association of β-lactamase with the OM. Depending on the location of the β-lactamase in the membrane, the presence of β-lactamase in the OM in conjunction with reduced OM permeability may make the cell especially resistant to the diffusion of β-lactam antibiotics. As well as being trapped or hydrolysed by β-lactamase in the periplasmic cavity of the cell (the cell's major line of defence), β-lactams may be trapped or hydrolysed by β-lactamase in the OM preventing them from reaching their target PBPs.

The β-lactamases of the Gram-negative bacteria are thought to be solely periplasmic. However, the occurrence of a small proportion of β-lactamase in the OM has been postulated from studies on the rate of hydrolysis of β-lactams by whole cells (Nichols, 1987) and discrepancies shown in the the Zimmermann-Rosselet assay (Zimmermann and Rosselet, 1977) for *P. aeruginosa*, where the permeability parameter (C) was found to be dependent upon the concentration of β-lactam used in the assay (Hewinson *et al.*, 1986). There is also evidence of other penicillin recognising enzymes being located in the OM e.g. Booth and Curtis (1977) found levels of carboxypeptidase in *P. aeruginosa* PAO1 OMs prepared by sucrose density gradient centrifugation, Shafer and Judd (1991) recently identified the conserved 44 kD OM protein of *Neisseria gonorrhoeae* as PBP3 (and suggested that PBP3 located in the OM might protect the essential PBPs from interaction with β-lactams), enzymes involved

with peptidoglycan synthesis in the OM of Gram-negative bacteria have been reported elsewhere, and examples of membrane-bound hydrophobic β-lactamases have been found in Gram-positive bacteria (Nielsen and Lampen, 1982b; 1983).

The results show that in this particular preparation approximately 1% of the β-lactamase activity is located in the OM. This is the first direct demonstration of such a location and the value of 1% compares well with that of 5% predicted for *E. coli* by Liu and Nikaido (1991). However, the results in chapter 4 show that in some strains up to 20% of the enzyme activity of the cell may be associated with the OM material. The existence of this enzyme may partly explain some of the discrepancies in permeability found in the Zimmermann-Rosselet permeability assays. This also suggests that OM-bound β-lactamase alone cannot be responsible for the discrepancies seen with *P. aeruginosa* strains and the Zimmermann-Rosselet permeability assay because good results have been obtained with *E. coli* strains, which also contain OM-bound β-lactamase

The β-lactamase activity in the OM may be due to enzyme that is in the process of being excreted by the cell or possibly, is actually located in the OM. In this preparation (*P. aeruginosa* SAI^{const}) the majority of the β-lactamase activity was associated with the second major peak (Sample B) in the protein sedimentation profile, which on SDS-PAGE was shown to contain mostly OM material. This fraction contained 0.62% of the 1% found in the OM of this strain. Sample A corresponding to the first peak of the protein sedimentation profile contained the other major activity of the sample (0.22%) with similar amount being found in the third and fourth peaks- 0.01 and 0.03 respectively. Similar distributions were found in other strains which were tested.

Although it was not possible to show whether the enzyme is surface-exposed, and therefore able to hydrolyse antibiotic directly on the cell surface, it is possible that this mechanism could contribute to the overall resistance of the organism. Possible mechanisms could involve trapping and/or slow hydrolysis on the bacterial surface and interference with the penetration of the antibiotics through the porin channels in the OM. Further experiments could be conducted to see whether the β-lactamase sticks to LPS, which would help explain its OM location.

The results from section 5.3 suggest that the presence of β-lactamase detected in the cell wall represents a genuine observation and is not an artefact of preparation. Washing could not remove the membrane-bound β-lactamase from the membrane but it did remove semi-purified β-lactamase added to TMP preparations before and after breakage, so it is unlikely that OM-associated β-lactamase activity was due to the periplasmic enzyme sticking to the membrane. OM-associated β-lactamase did not appear to be due to an electrostatic interaction caused by the presence or sarkosyl because the β-lactamase occurs in non-sarkosyl prepared OMs, nor is it due to the formation of micelles by the OM during French pressing or sarkosyl treatment because no enzyme activity is seen in PAO1 (non-enzyme containing samples) OMPs to which β-lactamase has been added before and after breakage or added with sarkosyl after washing.

Section 5.4 shows that a significant amount of β-lactamase in the OM. In this location it might function to reduce the amount of β-lactam entering the cell (Nichols, 1987). Obviously, any mechanism that confers β-lactam resistance to the cell must be an advantage to it in order for it to proliferate *in vivo*.

Gram-negative organisms excrete a variety of proteins (Pugsley and Schwartz, 1985). An elaborate mechanism exists for the transport of certain proteins across the CM. Secreted proteins are localised either in the the OM or the periplasmic space and perform a variety of important functions such as nutrient transport and osmoregulation (Lugtenberg and Alphen, 1983; Hepple, 1971). Proteins that are destined for secretion are synthesised in precursor form i.e. they contain an amino acid extension consisting of approximately 20-30 amino acids (the signal sequence) at their amino terminal (Oliver, 1985). The terminal precursor form of a protein is able to traverse the inner membrane of the cell envelope. Once on the other side, the signal sequence is cleaved, and the mature form of the protein folds to its native conformation. However, the presence of the outer membrane prevents the release of proteins into the extracellular fluid.

One way for the protein to be excreted is to increase the permeability of the outer membrane to allow the polypeptide to diffuse into the culture medium. Georgiou *et al.* (1988) found that the induction of synthesis of the peripasmic enzyme β-lactamase in *E. coli* containing the plasmid ptac11 resulted in the excretion of 90% of the active

enzyme and partial excretion of other periplasmic proteins. They also showed that high levels of synthesis of β-lactamase were necessary for excretion and that the synthesis and export of the two outer membrane proteins (Omp A and OmpC) were effected by β-lactamase over-production.

In some cases the overproduction of a secreted protein jams the secretion machinery of the cell and inhibits the correct localisation of other exported proteins (Ito *et al.*, 1979; Josetsson and Randall, 1981; Koshland and Botstein, 1980; Bankaitis and Bassford, 1984). Hence, β-lactamase may become "misplaced" and located in the OM. The high level of β-lactamase synthesis might affect the processing of certain outer membrane proteins which could cause a non-specific leakage of periplasmic enzymes. (This could also lead to a transient location of the β-lactamase in the weakened OM.) Some workers have shown that OM proteins A and C were affected with their expression being decreased in the induced cells (Georgiou *et al.*, 1988). Release of periplasmic enzymes has been seen in *E. coli* mutants with defective OMs (Hancock, 1984). Activation of the *kil* gene has been shown to result in release of a penicillinase into the culture medium (Kobayashi *et al.*, 1986). Lazzaroni *et al.* (1985) showed that cells transformed with multicopy plasmids carrying the *pho*A gene and grown in alkaline medium released a large percentage of the alkaline phosphatase into the media. They reported that amplification of alkaline phosphatase synthesis was necessary for excretion as did Georgiou *et al.* (1988) with the production of β-lactamase. Georgiou *et al.* (1988) did not observe excretion in cells with low levels of β-lactamase production. The excretion of β-lactamase was also found to increase the sensitivity of the cells to detergents and cause the non-specific release of other periplasmic enzymes into the growth media. Production of high levels of β-lactamase might interfere with the synthesis of the cell envelope proteins and result in a subsequent change in OM permeability. Synthesis of export-defective proteins causes a generalised block of protein secretion (Oliver, 1985; Josetsson and Randall, 1981; Bankaitis and Bassford, 1984). This could explain why some β-lactamase is found in the OM.

Bowden and Georgiou (1990) with their experiments with TEM β-lactamase in *E. coli* showed that under conditions of high expression the β-lactamase precursor accumulated in the cell cytoplasm, probably because a certain component of the export machinery became limiting. The precursor fractionated with the insoluble fraction of

the OM so it was presumed to be either present in high molecular weight complex formed either by self-association or by binding to the CM. However, the β-lactamase precursor did not migrate with the membrane fraction when a sucrose density gradient was used to separate aggregated proteins from membranes.

The amino-terminal extension of the *B. licheniformis* β-lactamase has a lipoprotein modification which is very similar to the *E. coli* OM lipoprotein amino-terminus. This modification consists of a diacyl glyceride group in thioether linkage to an amino-terminal cysteine residue, and a third long chain fatty acid in amide linkage, blocking the amino terminus to Edman sequencing. Nielsen and Lampen (1982a) showed, using radiolabelled palmitate and SDS PAGE electrophoresis, that *S. aureus* Types A and C and *B. cereus* type I membrane-bound penicillinases carry the same type of glyceride thioether modification. The Gram-positive set of penicillinases within the class A β-lactamases are also further related in possessing signal peptides which allow both secretion and covalent modification for membrane attachment. The secreted and membrane bound forms of the *B. licheniformis* 749 and *S. aureus* enzymes are known to be products of single genes (although this is not absolutely certain for *B. cereus,* as the exo-form and the membrane-form have been shown to be immunologically distinct but have identical substrate specificities) (Abraham and Waley, 1979). Comparison of the whole amino acid sequences shows that certain structural elements are required for the addition of a glyceride modification. The secreted form of the enzyme lacks the cysteine residue and covalently linked long chain fatty acid (Neilsen and Lampen, 1983).

Comparison of the amino acid sequences of the signal peptides of *S. aureus* Type A penicillinase, *B. licheniformis* β-lactamase, Gram-negative OM proteins and the amino acid sequences of a number of bacterial signal peptides that can accept a glyceride thioether show a number of similarities. In the signal peptide of *S. aureus* Type A penicillinase the critical membrane-anchoring cysteine is surrounded by an amino acid sequence which is homologous to the amino acid sequence surrounding the cysteine in both *B. licheniformis* β-lactamase and the Gram-negative OM proteins signal peptides. The *S. aureus* Type A penicillinase, *B. licheniformis* β-lactamase and the Gram-negative OM proteins contain hydrophobic stretches which include the modified cysteine residues and are considered to be the segment spanning the membrane in any of the

membrane insertion and secretion models. The *S. aureus* penicillinase has a positively charged residue adjacent to the initiator methionine, which is a common feature to all bacterial signal sequences. The main differences are that this charged region in *S. aureus* is smaller than in *B. licheniformis* and the long hydrophilic segment between the amino-terminus of the membrane form and the secreted exo-form is not present in *S. aureus*. However, a small hydrophilic portion has been preserved. This could be the smallest allowable distance between the hydrophobic membrane anchorage point and the globular body of the protein.

Two possible explanations for exocellular β-lactamase must be considered. The first is that a proportion of the cells lyse during culture and/or harvesting. *P. aeruginosa* is particularly sensitive to cold shock and periplasmic β-lactamase can be released in this way (Lei *et al.*, 1991). In these experiments the cultures were centrifuged at 20°C to harvest cells and prepare culture supernatants. Control experiments showed no significant difference in enzyme levels from cells harvested at 20°C or 37°C. This suggests that the enzyme measured in the culture supernatants represented material genuinely exported during culture. Secondary structural predictions for the amino acid sequence of the mature chromosomal β-lactamase (Lodge *et al.*, 1990) show that there is a strong propensity for the 20 amino acid residues on the amino-terminus to form an α-helix (after removal of the peptide leader sequence). This structural motif could constitute an OM-spanning helix involved in the "secretion" or transport of the β–lactamase across the outer membrane. Helical wheel projection of the amino-terminal segment of the mature β-lactamase (AGEAPADRLKALVDAAEQ) reveals an amphipathic α-helix, with a pronounced division between the hydrophobic face (approximately two thirds) and the hydrophilic face (approximately one third of the helix) (Figure 10). Amphipathic helices are recognised to be membrane-seeking structures (Eisenberg *et al.*, 1982). Similar features have been identified in known secretory proteins in *P. aeruginosa*, including exotoxin A, elastase, alkaline phosphatase and phospholipase C (Lazdunski *et al.*, 1990). β-Lactamase might therefore be considered together with alkaline phosphatase and phospholipase C, in that it is active both in the periplasm and is released into the medium, with a small amount located in the OM.

The high levels of exocellular β-lactamase observed in *P. aeruginosa* strains *in vitro* agree with the high levels observed in sputum from CF patients infected with *P. aeruginosa* (Giwercman *et al.*, 1991) and probably represent genuine extracellular enzyme. The export of the enzyme may represent a defence mechanism of the organism *in vivo* rather than a secondary consequence of cell lysis. The nature of the inducing β-lactam and the proposed biofilm or microcolony mode of growth in the lung also affect the amount of enzyme expressed (Giwercman *et al.*, 1991). Bacterial usually produce a capsule when they are in a condition of nutrient starvation. Mucoid *P. aeruginosa* usually appear after colonisation of the human CF lung by non-mucoid types which might imply that the bacterium is experiencing a state of nutritional starvation and has had to switch to the mucoid phenotype to survive (Pedersen *et al.*, 1990). The capsule functions as a sieve or trap that concentrates nutrients essential for the existence of the bacteria (Costerton *et al.*, 1981). Giwercman *et al.* (1991) suggested, from their observations with monoclonal antibodies raised against the enzyme, that the mucoid exopolysaccharide concentrated the β-lactamase around the cell. The capsule prevents the β-lactamase from diffusing into the surrounding medium and ideally locates it to protect the cells from β-lactams (Giwercman *et al.*, 1991). (It has been hypothesised that a possible role of the exopolysaccharide may be to modulate the function of exoenzymes by binding them, concentrating them at distinct sites in the lung tissue and slowing their release into the environment and possibly that exoenzyme expression is influenced by alginate (Ohman and Goldberg, 1990). Perhaps a similar effect on β-lactamase production occurs.)

CHAPTER 6

6. INHIBITION KINETICS OF *P. AERUGINOSA* SAI^{const}, *S. AUREUS* PC1 AND *E. CLOACAE* P99 β-LACTAMASES.

6.1 Aims.

The aims of this section of work were:

(1) to determine whether the inhibition of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 β-lactamases with tazobactam and a number of classical inhibitors (ampicillin, cloxacillin and cefotaxime) was progressive, reversible or irreversible.

(2) to determine the type of inhibition kinetics (i.e. competitive, non-competitive or uncompetitive) exhibited by the purified β-lactamase of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 β-lactamase with inhibitors that exhibited reversible inhibition. Values for the K_m, V_{max} and K_i for each enzyme with each inhibitor were determined and compared;

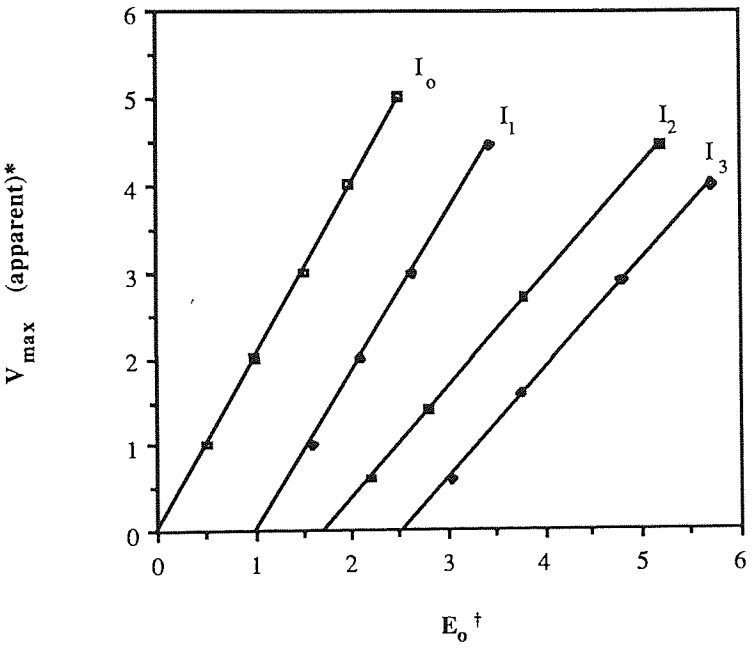
(3) to determine the active site residues involved in the binding of tazobactam to *P. aeruginosa* SAI^{const} β-lactamase.

6.2 Determination of reversibility or irreversibility of inhibition.

An investigation was carried out to determine whether the inhibition of each enzyme with each inhibitor was reversible or irreversible.

The effect of a covalent, irreversible inhibitor is a reduction of the amount of active enzyme present, which results in a reduction of V_{max} (apparent). The easiest way to distinguish between reversible and irreversible inhibition is to plot V_{max} against E_o. For a non-competitive reversible inhibitor, the slope of the plot will decrease from the control and go through the origin. In the case of an irreversible inhibitor, the plot will have a finite intercept on the E_o axis that corresponds to the amount of inhibitor reacting with the covalent inhibitor.

6.2.1 Reversibility or irreversibility of inhibition of P. aeruginosa SAI^{const} β-lactamase with tazobactam. Figure 11 shows the plot of V_{max} against E_o to detect irreversible inhibition for the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} by tazobactam. A range of enzyme concentrations were pre-incubated with a fixed concentration of tazobactam. The reaction was then initiated by addition of a fixed concentration of substrate (nitrocefin). The reaction rate was then measured by measuring the change in absorbance at 492nm. The procedure was



* change in absorbance units at 492nm /min/mg protein x 10^3
† μg protein /ml

Figure 11. Plot of V_{max} against E_o to detect irreversible inhibition of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} by tazobactam. Plot of V_{max} against E_o to determine irreversible inhibition for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with tazobactam. Fixed inhibitor concentrations (I_1-I_3) were incubated with four different enzyme concentrations for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. V_{max} was determined for each reaction and plotted against E_o (enzyme concentration). I_o = reaction with no inhibitor present, $I_1 = 8 \times 10^{-2}$ μg/ml tazobactam, $I_2 = 10 \times 10^{-2}$ μg/ml tazobactam, $I_3 = 12 \times 10^{-2}$ μg/ml tazobactam and $I_4 = 14 \times 10^{-2}$ μg/ml tazobactam.

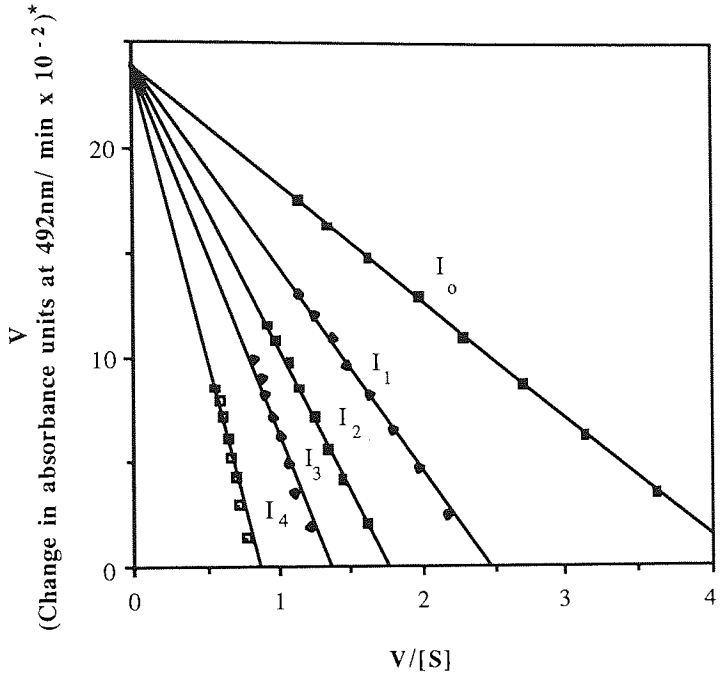
repeated for several different concentration of tazobactam. The V_{max} for each reaction was calculated and plotted against enzyme concentration (E_0).

The graph shows that the inhibition of *P. aeruginosa* SAI^{const} β-lactamase by tazobactam was progressive and irreversible. I_0 showed the effect of increasing enzyme concentration on the uninhibited reaction. The rate of the enzyme catalysed reaction was directly proportional to the concentration of enzyme present when the substrate was in excess. This relationship held true for I_0 (at this substrate concentration) in which there was a linear increase in V_{max} as E_0 was increased and the plot passed through the graph origin. The same relationship would apply for the inhibited reaction if tazobactam was a reversible non-competitive inhibitor, the slope of the plot would decrease from the control because $V_{max}(\text{apparent}) = (k_{cat}\ E_0/(1+I_0/K_i))$ and pass through the origin of the graph. In the case of the tazobactam inhibited reaction (I_1-I_3) the plots did not pass through the origin but intercepted the E_0 axis at a point which must correspond to the amount of enzyme reacting with the inhibitor. This result is characteristic of an irreversible inhibitor which may form a covalent bond or bind very tightly to the enzyme. This suggests that tazobactam is an irreversible inhibitor of *P. aeruginosa* SAI^{const} chromosomal β-lactamase. This would also suggest that tazobactam binds directly to the active site of the enzyme and favours the use of the Knowles reaction mechanism for docking the inhibitor into the active site of the enzyme in the modelling studies.

A final check involved determining the type of kinetics exhibited by the enzyme with tazobactam without an initial enzyme-inhibitor pre-incubation period. The kinetic assay described in section 2.10.5 was carried out except that tazobactam was not pre-incubated with the enzyme but was added with the substrate instead. The results are shown in Figure 12.

Figure 12 shows that when tazobactam is not pre-incubated with the *P. aeruginosa* SAI^{const} β-lactamase it shows competitive inhibition kinetics. A competitive inhibitor is one which binds directly to the active site of the enzyme so the results suggests that tazobactam is binding directly to the active site of the enzyme. Initially tazobactam competes with the substrate for the binding site on the enzyme. Again this favours the use of the Knowles model for docking tazobactam into the active site in

modelling studies.

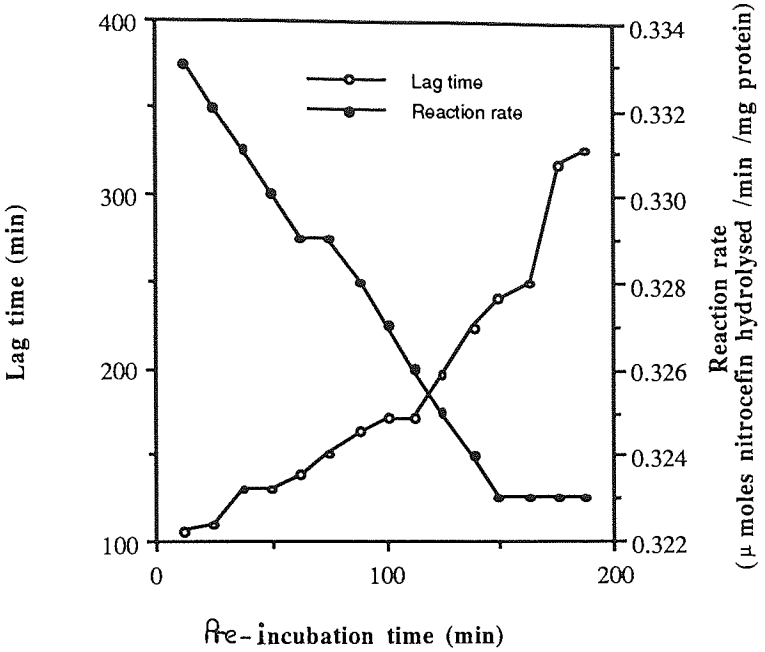


* per mg protein

Figure 12. Eadie-Hofstee plot for the inhibition of the *P. aeruginosa* SAI^{const} β-lactamase by tazobactam without pre-incubation with the inhibitor. Plot of V against V/[S] to determine inhibition type for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with tazobactam. Four different inhibitor concentrations (I_1-I_4) were mixed with nitrocefin (0.025 mg/ml) at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of the enzyme and the change in absorbance at 492nm followed over 5 min. I_0 = reaction with no inhibitor present, $I_1 = 8 \times 10^{-2}$ μg/ml tazobactam, $I_2 = 10 \times 10^{-2}$ μg/ml tazobactam, $I_3 = 12 \times 10^{-2}$ μg/ml tazobactam and $I_4 = 14 \times 10^{-2}$ μg/ml tazobactam.

6.2.2 Reversibility or irreversibility of inhibition of S. aureus β-lactamase with tazobactam. A series of experiments was conducted to determine the effects on reaction rate of different pre-incubation times with fixed inhibitor, substrate and enzyme concentrations. The results for the effect of pre-incubation time of the *S. aureus* PC1 β-lactamase with tazobactam is shown in Figure 13.

Figure 13 shows the effect of the length of pre-incubation with tazobactam on the rate of reaction of the *S. aureus* PC1 β-lactamase with nitrocefin.



* length of time between the addition of the inhibitor-enzyme mixture to nitrocefin and the start of the hydrolysis reaction shown by the change in absorbance of the solution at 492nm.

Figure 13. Effect of the length of pre-incubation time with tazobactam on the rate of reaction and lag* time of the β-lactamase of *S.aureus* PC1 with nitrocefin. A fixed concentration of *S. aureus* PC1 β-lactamase was incubated at 37°C, in 5 mM phosphate buffer (pH 7.0) with a fixed concentration of tazobactam for a range of different time periods (15-195 min). The reaction was then initiated by the addition of the enzyme-inhibitor mixture to a fixed substrate (nitrocefin) concentration. The reaction was then followed by monitoring the change in absorbance at 492nm in an Anthos plate reader.

The plot shows that for the reaction of *S. aureus* PC1 with tazobactam the rate of nitrocefin hydrolysis decreased with increasing length of pre-incubation time, however, the enzyme appeared to regain its activity. After the initial inhibition of the enzyme, shown by a pause (lag phase) between the addition of the enzyme-inhibitor mixture to the nitrocefin and the start of the reaction, the enzyme recovered its activity and started to hydrolyse the nitrocefin. The length of this lag phase was dependent on the length of the pre-incubation time. The longer the pre-incubation time then the longer the lag phase. This suggests that the inhibition of the *S. aureus* PC1 β-lactamase is time dependent and progressive but is also reversible.

A similar plot for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with

tazobactam showed that the rate of nitrocefin hydrolysis decreased with increasing length of pre-incubation time. However, unlike the *S. aureus* PC1 β-lactamase the enzyme did not regain its activity. After the addition of the enzyme-inhibitor mixture to the nitrocefin there was no lag phase like that seen with *S. aureus* PC1 β-lactamase. The *P. aeruginosa* SAI^{const} β-lactamase started to hydrolyse the nitrocefin immediately. This suggests that the inhibition of the *P. aeruginosa* SAI^{const} β-lactamase is time dependent, progressive and irreversible.

A similar plot for the reaction of *E. cloacae* P99 β-lactamase showed that when the pre-incubation time was increased beyond 200 min a slight lag phase was observed with *E. cloacae* P99 β-lactamase, perhaps then, there is some evidence of a partially reversible inhibition having taken place. Akora *et al.* (1989) observed partial reversibility of the inhibition of *E. cloacae* 100-con by tazobactam but commented that the inactivated complexes were extremely stable.

No lag phase was found with either the *P. aeruginosa* SAI^{const}, *E. cloacae* P99 or *S. aureus* PC1 β-lactamases with cloxacillin, ampicillin or cefotaxime.

6.3 Determination of type of inhibition for β-lactamases of *P. aeruginosa* SAI^{const}, *E. cloacae P99* and *S. aureus* PC1 with cloxacillin, ampicillin, cefotaxime and tazobactam.

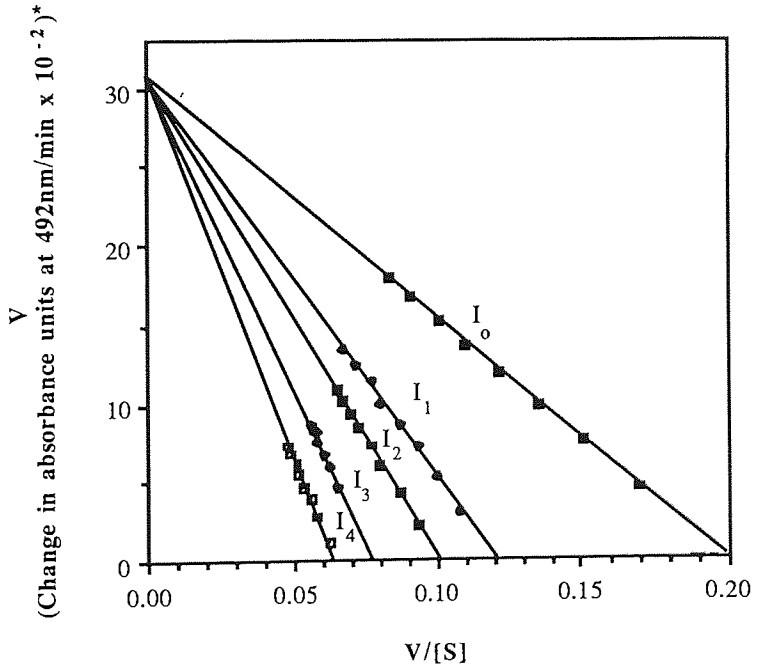
The reaction kinetics of each enzyme with each inhibitor, which displayed reversible inhibition kinetics in section 6.2, were examined using a range of substrate and inhibitor concentrations to determine the type of reversible inhibition. The results in Figures 14-22 show the Eadie-Hofstee plots obtained on reaction of each enzyme with each inhibitor (except for cefotaxime and ampicillin with *S.aureus* PC1 β-lactamase).

Micro-plate assays involving eight concentrations of substrate (nitrocefin) and four different concentrations of inhibitor and a control of sterile phosphate buffer were set up as described in section 2.12.2.

6.3.1. Reaction of P. aeruginosa SAI^{const} β-lactamase with cloxacillin, ampicillin and cefotaxime. The β-lactamase of *P. aeruginosa* SAI^{const} was pre-

incubated with differing concentrations of cloxacillin, ampicillin, cefotaxime and sterile phosphate buffer for 5 min. The reactions were then initiated by the addition of aliquots of the enzyme-inhibitor or enzyme-buffer mixture to separate aliquots of eight different concentrations of nitrocefin. The change in absorbance of the reaction mixture at 492nm was then monitored in an Anthos plate reader. The initial velocity (V) was then plotted against the initial velocity divided by substrate concentration (Eadie-Hofstee plot) and the K_m, V_{max} and K_i determined for the enzyme at each inhibitor concentration. The results are shown in Figures 14 to 16.

Figure 14 shows the Eadie-Hofstee plot for the inhibition of *P. aeruginosa* SAI^{const} β-lactamase by cloxacillin.

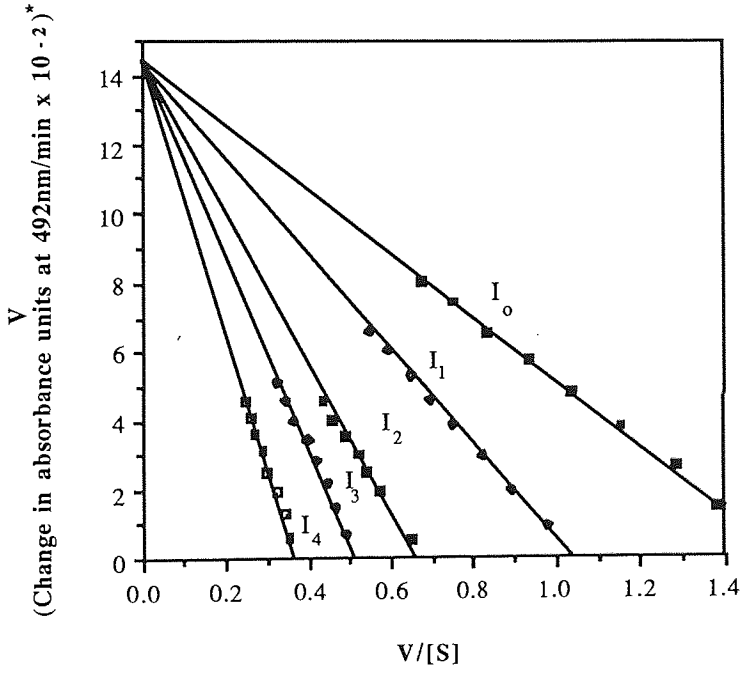


* per mg protein

Figure 14. Eadie-Hofstee plot for the inhibition of the *P. aeruginosa* SAI^{const} β-lactamase by cloxacillin. Plot of V against V/[S] to determine inhibition type for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with cloxacillin. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_0 = reaction with no inhibitor present, I_1 = 2.0 x10^{-3} μg/ml cloxacillin, I_2 = 4.0 x 10^{-3} μg/ml cloxacillin, I_3 = 8.0 x10^{-3}μg/ml cloxacillin and I_4 = 12.0 x10^{-3} μg/ml cloxacillin.

The graphs shows that cloxacillin acted as a competitive inhibitor of *P. aeruginosa* SAI^{const} β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that cloxacillin must bind to the active site of enzyme.

Figure 15 shows the Eadie-Hofstee plot for the inhibition of *P. aeruginosa* SAI^{const} β-lactamase by ampicillin.



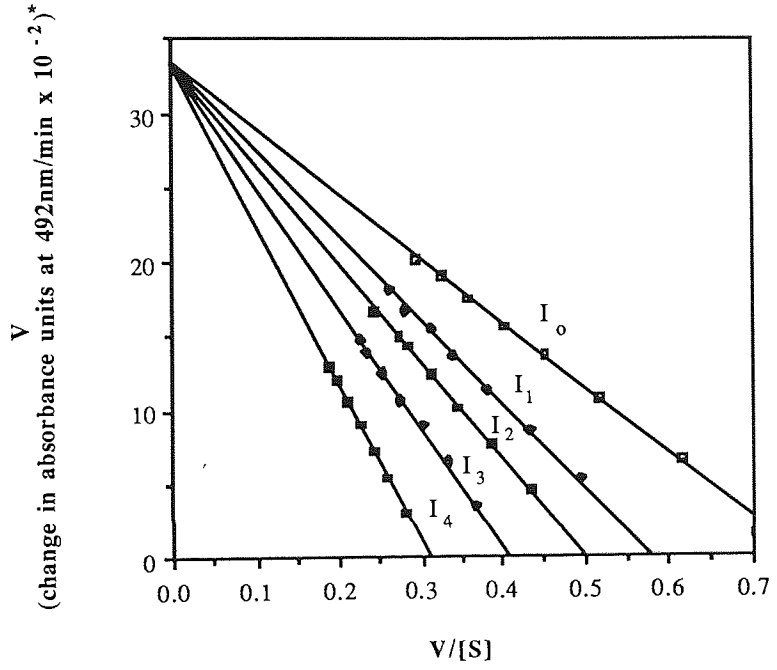
* per mg protein

Figure 15. Eadie-Hofstee plot for the inhibition of the *P. aeruginosa* SAI^{const} β-lactamase by ampicillin. Plot of V against V/[S] to determine inhibition type for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with ampicillin. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_o = reaction with no inhibitor present, $I_1 = 4 \times 10^{-1}$ μg/ml ampicillin, $I_2 = 8 \times 10^{-1}$ μg/ml ampicillin, $I_3 = 10 \times 10^{-1}$ μg/ml ampicillin and $I_4 = 12 \times 10^{-1}$ μg/ml ampicillin.

The graphs shows that ampicillin acted as a competitive inhibitor of *P. aeruginosa* SAI^{const} β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9

and 10. This indicates that ampicillin must bind to the active site of enzyme.

Figure 16 shows the Eadie-Hofstee plot for the inhibition of *P. aeruginosa* SAI^{const} β-lactamase by cefotaxime.



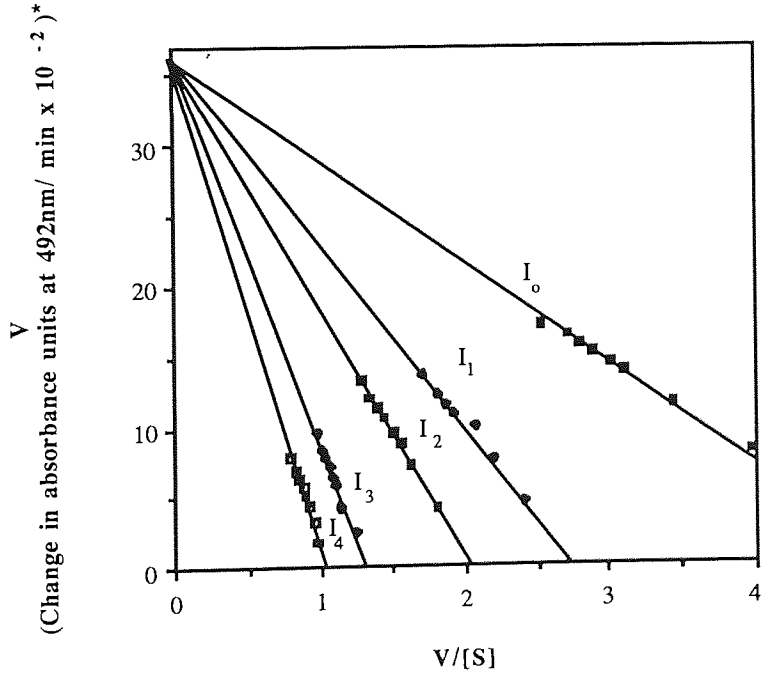
* per mg protein

Figure 16. Eadie-Hofstee plot for the inhibition of the *P. aeruginosa* SAI^{const} β-lactamase by cefotaxime. Plot of V against V/[S] to determine inhibition type for the reaction of *P. aeruginosa* SAI^{const} β-lactamase with cefotaxime. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_0 = reaction with no inhibitor present, I_1 = 2 x10^{-2} μg/ml cefotaxime, I_2 = 4 x 10^{-2} μg/ml cefotaxime, I_3 = 8 x10^{-2} μg/ml cefotaxime and I_4 = 12 x10^{-2} μg/ml cefotaxime.

The graphs shows that cefotaxime acted as a competitive inhibitor of *P. aeruginosa* SAI^{const} β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that cefotaxime must bind to the active site of enzyme.

6.3.2. Reaction of S.aureus PC1 β-lactamases with cloxacillin, and tazobactam. The β-lactamase of *S.aureus* PC1 was pre-incubated with differing concentrations of cloxacillin, tazobactam and sterile phosphate buffer for 5 minutes. The reactions were then initiated by the addition of aliquots of the enzyme-inhibitor or enzyme-buffer mixture to separate aliquots of eight different concentrations of nitrocefin. The change in absorbance of the reaction mixture at 492nm was then monitored in an Anthos plate reader. The initial velocity (V) was then plotted against the initial velocity divided by substrate concentration (Eadie-Hofstee plot) and the K_m, V_{max} and K_i determined for the enzyme at each inhibitor concentration. The results are shown in Figures 17 to 18.

Figure 17 shows the Eadie-Hofstee plot for the inhibition of *S. aureus* PC1 β-lactamase by cloxacillin.

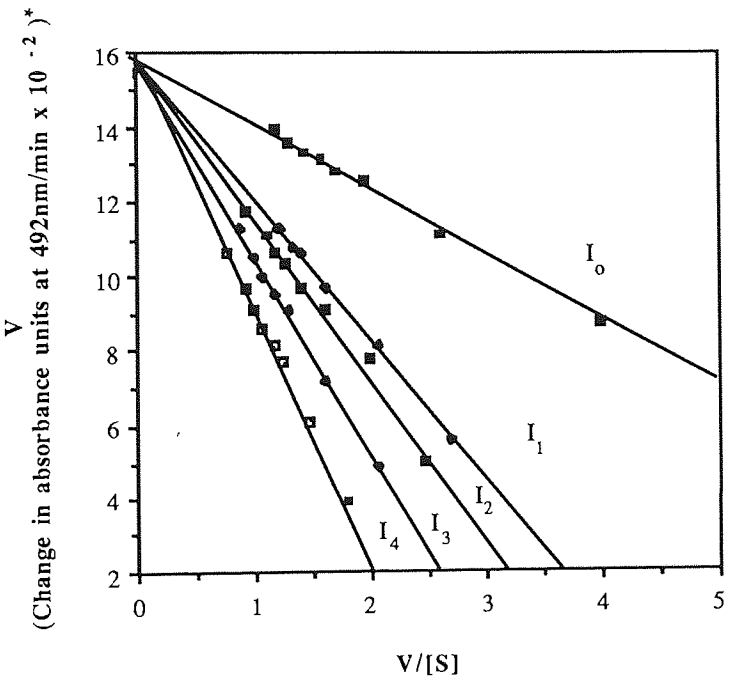


* per mg protein

Figure 17. Eadie-Hofstee plot for the inhibition of the *S. aureus* PC1 β-lactamase by cloxacillin. Plot of V against V/[S] to determine inhibition type for the reaction of *S. aureus* PC1 β-lactamase with cloxacillin. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_o = reaction with no inhibitor present, $I_1 = 8.0 \times 10^{-3}$ µg/ml cloxacillin, $I_2 = 10 \times 10^{-3}$ µg/ml cloxacillin, $I_3 = 12 \times 10^{-3}$ µg/ml cloxacillin and $I_4 = 14 \times 10^{-3}$ µg/ml cloxacillin

The graphs shows that cloxacillin acted as a competitive inhibitor of *S. aureus* PC1 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that cloxacillin must bind to the active site of enzyme.

Figure 18 shows the Eadie-Hofstee plot for the inhibition of *S. aureus* PC1 β-lactamase by tazobactam.



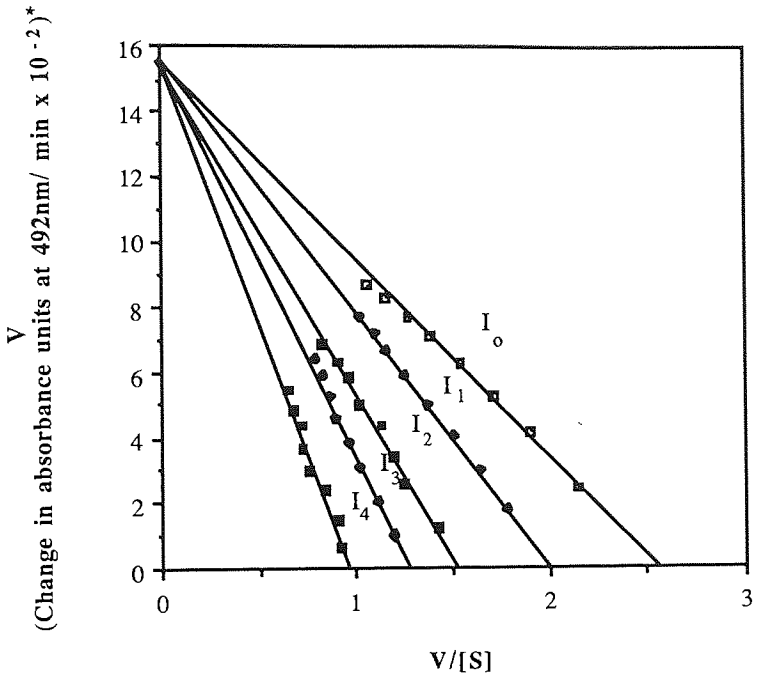
* per mg protein

Figure 18. Eadie-Hofstee plot for the inhibition of the *S. aureus* PC1 β-lactamase by tazobactam.Plot of V against V/[S] to determine inhibition type for the reaction of *S. aureus* PC1 β-lactamase with tazobactam. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. Io = reaction with no inhibitor present, $I_1 = 8 \times 10^{-2}$ µg/ml tazobactam, $I_2 = 12 \times 10^{-2}$ µg/ml tazobactam, $I_3 = 16 \times 10^{-2}$ µg/ml tazobactam and $I_4 = 20 \times 10^{-2}$ µg/ml tazobactam.

The graphs shows that tazobactam acted as a competitive inhibitor of *S. aureus* PC1 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that tazobactam must bind to the active site of enzyme

6.3.3. Reaction of E. cloacae P99 β-lactamases with cloxacillin, ampicillin, cefotaxime and tazobactam. The β-lactamase of *E. cloacae* P99 was pre-incubated with differing concentrations of cloxacillin, ampicillin, cefotaxime, taxobactam and sterile phosphate buffer for 5 min. The reactions were then initiated by the addition of aliquots of the enzyme-inhibitor or enzyme-buffer mixture to separate aliquots of eight different concentrations of nitrocefin. The change in absorbance of the reaction mixture at 492nm was then monitored in an Anthos plate reader. The initial velocity (V) was then plotted against the initial velocity divided by substrate concentration (Eadie-Hofstee plot) and the K_m, V_{max} and K_i determined for the enzyme at each inhibitor concentration. The results are shown in Figures 19 to 22.

Figure 19 shows the Eadie-Hofstee plot for the inhibition of *E. cloacae* P99 β-lactamase by cloxacillin.

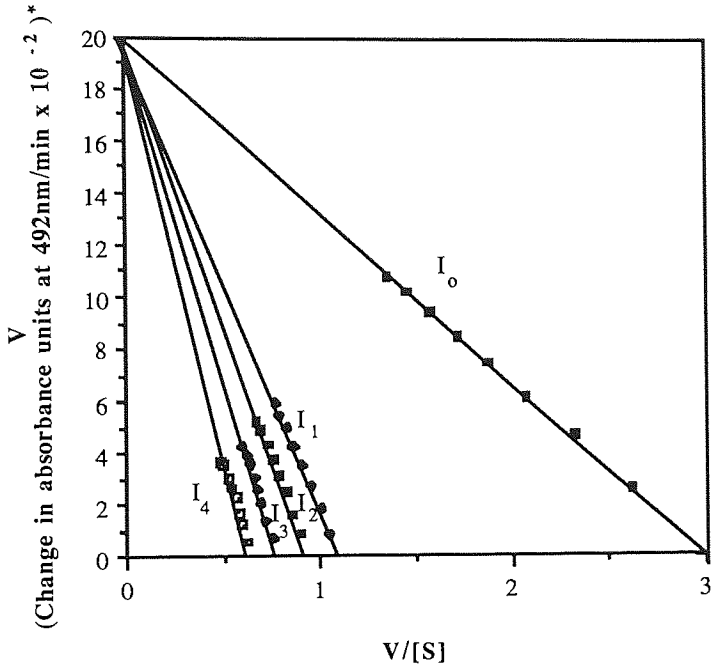


* per mg protein

Figure 19. Eadie-Hofstee plot for the inhibition of the *E.cloacae* P99 β-lactamase by cloxacillin. Plot of V against V/[S] to determine inhibition type for the reaction of *E. cloacae* P99 β-lactamase with cloxacillin. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_o = reaction with no inhibitor present, $I_1 = 10 \times 10^{-2}$ µg/ml cloxacillin, $I_2 = 12 \times 10^{-2}$ µg/ml cloxacillin, $I_3 = 14 \times 10^{-2}$ µg/ml cloxacillin and $I_4 = 16 \times 10^{-2}$ µg/ml cloxacillin.

The graphs shows that cloxacillin acted as a competitive inhibitor of *E. cloacae* P99 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that cloxacillin must bind to the active site of enzyme.

Figure 20 shows the Eadie-Hofstee plot for the inhibition of *E. cloacae* P99 β-lactamase by ampicillin.

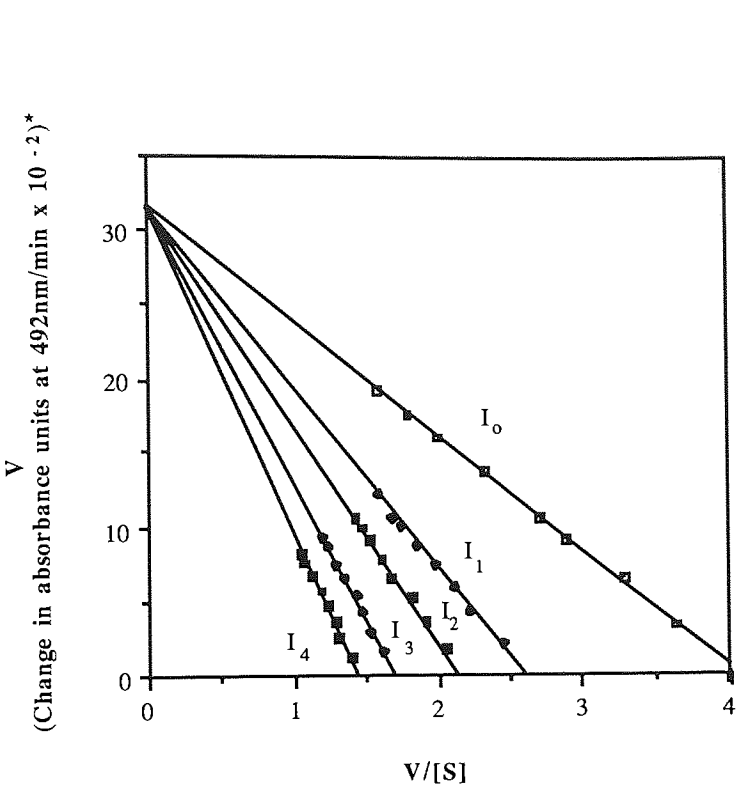


* per mg protein

Figure 20. Eadie-Hofstee plot for the inhibition of the *E. cloacae* P99 β-lactamase by ampicillin. Plot of V against V/[S] to determine inhibition type for the reaction of *E. cloacae* P99 β-lactamase with ampicillin. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_0 = reaction with no inhibitor present, I_1 = 8 x10^{-1} µg/ml ampicillin, I_2 = 10 x 10^{-1} µg/ml ampicillin, I_3 = 12 x10^{-1} µg/ml ampicillin and I_4 = 14 x10^{-1} µg/ml ampicillin.

The graphs shows that ampicillin acted as a competitive inhibitor of *E. cloacae* P99 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that ampicillin must bind to the active site of enzyme.

Figure 21 shows the Eadie-Hofstee plot for the inhibition of *E. cloacae* P99 β-lactamase by cefotaxime.

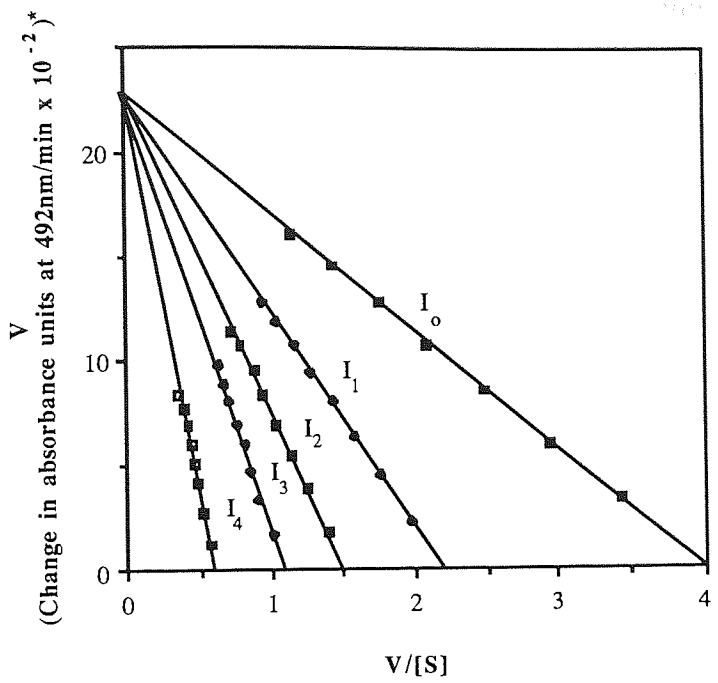


* per mg protein

Figure 21. Eadie-Hofstee plot for the inhibition of the *E.cloacae* P99 β-lactamase by cefotaxime. Plot of V against V/[S] to determine inhibition type for the reaction of *E. cloacae* P99 β-lactamase with cefotaxime. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_o = reaction with no inhibitor present, I_1 = 10 x 10^{-3} µg/ml cefotaxime, I_2 = 12 x 10^{-3} µg/ml cefotaxime, I_3 = 14 x 10^{-3} µg/ml cefotaxime and I_4 = 16 x 10^{-3} µg/ml cefotaxime.

The graphs shows that cefotaxime acted as a competitive inhibitor of *E. cloacae* P99 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that cefotaxime must bind to the active site of enzyme.

Figure 22 shows the Eadie-Hofstee plot for the inhibition of *E. cloacae* P99 β-lactamase by tazobactam.



* per mg protein

Figure 22. Eadie-Hofstee plot for the inhibition of the *E.cloacae* P99 β-lactamase by tazobactam. Plot of V against V/[S] to determine inhibition type for the reaction of *E. cloacae* P99 β-lactamase with tazobactam. The enzyme was incubated with four different inhibitor concentrations (I_1-I_4) for 5 min at pH 7.0 and 37°C in 5 mM phosphate buffer, the reaction initiated by the addition of nitrocefin (0.025 mg/ml) and the change in absorbance at 492nm followed over 5 min. I_o = reaction with no inhibitor present, $I_1 = 8 \times 10^{-2}$ μg/ml tazobactam, $I_2 = 10 \times 10^{-2}$ μg/ml tazobactam, $I_3 = 12 \times 10^{-2}$ μg/ml tazobactam and $I_4 = 14 \times 10^{-2}$ μg/ml tazobactam.

The graphs shows that tazobactam acted as a competitive inhibitor of *E. cloacae* P99 β-lactamase. Values of K_m, V_{max} and K_i are shown in Tables 9 and 10. This indicates that tazobactam must bind to the active site of enzyme.

6.4. Calculation of K_m and V_{max} values for reaction of *P. aeruginosa* SAI^{const}, *S.aureus* PC1 and *E. cloacae* P99 β-lactamases with nitrocefin inhibited by cloxacillin, ampicillin, cefotaxime and tazobactam.

For the reactions showing competitive kinetics the K_m and V_{max} values were calculated from the Eadie-Hofstee plots taking the intercept on the V axis to be equivalent to V_{max} for both inhibited and uninhibited reactions and either the slope of the line to be equivalent to $-K_m$ for reaction with no inhibitor present and $-K_m (1+ ([I_o] / K_i))$ for the reactions with inhibitor present or the intercept on the V/[S} axis to be equivalent to K_m/V_m for the uninhibited reaction and $V_m / K_m (1+ ([I_o] / K_i))$ for the inhibited reactions, from which the values of K_m and K_i could be calculated. The values of K_m and V_{max} are shown in Table 9 and the values of K_i are shown in Table 10.

Table 9. K_m and V_{max} values for the reaction of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 β-lactamases with nitrocefin inhibited by cloxacillin, ampicillin, cefotaxime and tazobactam.

CLOXACILLIN	*P. aeruginosa* SAI^{const}			*E. cloacae* P99			*S. aureus* PC1	
Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)
I_o 0	1.9	31.5×10^{-2}	I_o 0	0.1	15.5×10^{-2}	I_o 0	0.35	23.5×10^{-2}
I_1 2×10^{-3}	7.2	31.0×10^{-2}	I_1 10×10^{-2}	0.12	14.0×10^{-2}	I_1 8×10^{-3}	1.12	23.5×10^{-2}
I_2 4×10^{-3}	8.4	30.0×10^{-2}	I_2 12×10^{-2}	0.12	15.0×10^{-2}	I_2 10×10^{-3}	1.13	23.5×10^{-2}
I_3 8×10^{-3}	11.5	31.5×10^{-2}	I_3 14×10^{-2}	0.15	15.5×10^{-2}	I_3 12×10^{-3}	1.15	23.5×10^{-2}
I_4 12×10^{-3}	16	31.1×10^{-2}	I_4 16×10^{-2}	0.19	14.5×10^{-2}	I_4 14×10^{-3}	1.19	23.5×10^{-2}

AMPICILLIN	*P. aeruginosa* SAI^{const}			*E. cloacae* P99			*S. aureus* PC1	
Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)
I_o 0	1.9	34.5×10^{-2}	I_o 0	0.11	20.0×10^{-2}	I_o 0	0.3	30.5×10^{-2}
I_1 4×10^{-1}	2.0	33.5×10^{-2}	I_1 8×10^{-1}	0.47	19.5×10^{-2}	I_1	NM	NM
I_2 8×10^{-1}	2.4	34.0×10^{-2}	I_2 10×10^{-1}	0.55	19.5×10^{-2}	I_2	NM	NM
I_3 10×10^{-1}	3.05	34.0×10^{-2}	I_3 12×10^{-1}	0.65	19.5×10^{-2}	I_3	NM	NM
I_4 12×10^{-1}	4.8	33.5×10^{-2}	I_4 14×10^{-1}	0.80	20.0×10^{-2}	I_4	NM	NM

Table 9. Continued.

CEFOTAXIME	*P. aeruginosa* SAI^{const}			*E. cloacae* P99			*S. aureus* PC1	
Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)
I_o 0	1.8	37.0×10^{-2}	I_o 0	0.1	32.0×10^{-2}	I_o 0	0.3	23.0×10^{-2}
I_1 2×10^{-2}	2.2	36.0×10^{-2}	I_1 10×10^{-3}	0.51	31.0×10^{-2}	I_1	NM	NM
I_2 4×10^{-2}	2.6	35.5×10^{-2}	I_2 12×10^{-3}	0.48	30.5×10^{-2}	I_2	NM	NM
I_3 8×10^{-2}	3.6	37.0×10^{-2}	I_3 14×10^{-3}	0.54	32.0×10^{-2}	I_3	NM	NM
I_4 12×10^{-2}	4.8	37.0×10^{-2}	I_4 16×10^{-3}	0.60	31.0×10^{-2}	I_4	NM	NM

TAZOBACTAM	*P. aeruginosa* SAI^{const}			*E. cloacae* P99			*S. aureus* PC1	
Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)	Inhibitor Concentration µg/ml	K_m* (µM)	V_{max}† (µM/min)
I_o 0	2.0	33.0×10^{-2}	I_o 0	0.1	23.5×10^{-2}	I_o 0	0.32	15.8×10^{-2}
I_1	NM	NM	I_1 8×10^{-2}	0.45	22.0×10^{-2}	I_1 8×10^{-2}	0.45	15.2×10^{-2}
I_2	NM	NM	I_2 10×10^{-2}	0.50	22.5×10^{-2}	I_2 12×10^{-2}	0.50	14.5×10^{-2}
I_3	NM	NM	I_3 12×10^{-2}	0.62	23.5×10^{-2}	I_3 16×10^{-2}	0.62	15.5×10^{-2}
I_4	NM	NM	I_4 14×10^{-2}	0.75	22.5×10^{-2}	I_4 20×10^{-2}	0.72	15.5×10^{-2}

NM not measured
* Apparent K_m (µM), † Apparent V_{max} (µM/min)

The K_m and V_{max} for the reaction of the β-lactamases of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 with nitrocefin inhibited by cloxacillin, ampicillin, cefotaxime and tazobactam were determined from the Eadie-Hofstee plots.

The graphical evaluation (Eadie-Hofstee plots) resulted in a competitive type of inhibition for the reaction of *E. cloacae* P99 β-lactamase with nitrocefin after pre-incubation of the enzyme with each of the inhibitors (cloxacillin, ampicillin, cefotaxime and tazobactam). Competitive inhibition was also observed for the reaction of *S. aureus* PC1 β-lactamase with nitrocefin after pre-incubation of the enzyme with cloxacillin, cefotaxime and tazobactam. The reaction of *P. aeruginosa* SAI^{const} β-lactamase with nitrocefin after pre-incubation of the enzyme with cloxacillin, cefotaxime and ampicillin also displayed competitive reaction kinetics. The values of K_m for the enzymes with each of the inhibitors changes whilst the values of V_{max} remain unchanged. The effects on K_m and V_{max} are characteristic of competitive inhibition in which the inhibitor binds directly to the active site of the enzyme so preventing the binding of a substrate molecule. There is direct competition between the binding of substrate molecules and inhibitor molecules to the enzyme active site. This suggests that in any modelling studies tazobactam should be docked into the active site of the enzymes according to the Knowles model (Knowles, 1985).

6.5 Determination of K_i values for the reaction of *P. aeruginosa* SAI^{const}, *E.cloacae P99* and *S. aureus* PC1 β-lactamase with nitrocefin inhibited by cloxacillin, ampicillin, cefotaxime and tazobactam.

The K_i values for the reaction of the three β-lactamases with nitrocefin inhibited by each of the inhibitors was determined graphically from the Eadie-Hofstee plots (V against V/S) shown in Figures 14 to 22. The K_i for each inhibitor which showed competitive kinetics was calculated from the point of interception of the lines on the V/S axis which gives a value for $-K_i$ of $V_{max} / (K_m (1+ ([I]/K_i))$ and the slope which gives a value for $-K_m (1+([I]/K_i))$.

The reaction of *E. cloacae* P99, *P. aeruginosa* SAI^{const} and *S. aureus* PC1 enzymes with nitrocefin after inhibition with cloxacillin, ampicillin and cefotaxime showed pronounced competitive reaction kinetics. *S. aureus* PC1 and *E. cloacae* P99 β-lactamases also exhibit competitive reaction kinetics with tazobactam No results were obtained for *S. aureus* PC1 β-lactamase and cefotaxime as a chromophore was formed which masked the changes occurring in absorbance of the nitrocefin solution at 492nm.

Table 10. K_i values for the reaction of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 β-lactamases with nitrocefin after inhibition with cloxacillin, ampicillin, cefotaxime and tazobactam.

β-lactamase	K_i (μM)			
	Cloxacillin	Ampicillin	Cefotaxime	Tazobactam
P. aeruginosa SAI^{const}	0.015	0.37	0.14	-
E. cloacae P99	0.075	0.002	0.0018	0.0005
S. aureus PC1	0.002	-	-	0.005

The K_i for the reaction of the β-lactamases of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 with nitrocefin after inhibition with cloxacillin, ampicillin, cefotaxime and tazobactam were determined from an average of the values obtained with the Eadie-Hofstee plots of V against V/[S].

6.6 Determination of active site residues of *P. aeruginosa* SAI^{const} β-lactamase.

Irreversible inhibitors are useful in the investigation of the active site of an enzyme, since the inhibitor, unlike the substrate, will remain firmly bound to one of the amino-acids of the enzyme and thus act as a marker to enable the residues to be identified. The previous sections have demonstrated that tazobactam may bind irreversibly to the active site of the *P. aeruginosa* SAI^{const} β-lactamase. As an irreversible inhibitor, tazobactam could be used to identify the residues of the active site involved in the reaction by peptide fragment analysis experiments

6.6.1 Peptide fragmentation pattern of tazobactam inhibited P. aeruginosa SAI^{const} β-lactamase. Peptide fragments were prepared as described in section 2.16 and separated on a C18 reverse phase silica column using a gradient elution system. The elution profiles from the tryptic digests of tazobactam inhibited *P. aeruginosa* SAI^{const} β-lactamase were compared to the uninhibited *P. aeruginosa* SAI^{const} β-lactamase control and to trypsin alone to identify any peaks unique to the inhibited enzyme.

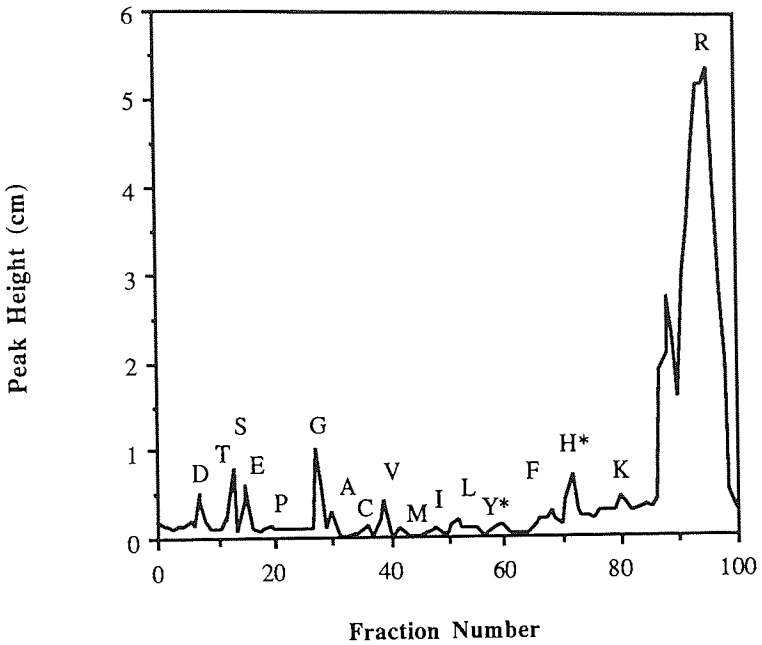
Reverse phase HPLC of the tryptic digests of the inhibited and uninhibited

enzymes monitored at 230nm revealed that a single peak was the only differences between the two peptide profiles. In all other regions of the peptide elution profile there were no differences.

These fractions were collected and used for amino acid analysis to identify the residues involved in the binding of tazobactam to the active site.

6.6.2 Amino acid analysis of tryptic digest of tazobactam-inhibited P. aeruginosa SAI^{const} β-lactamase.

The fractions corresponding to the only region of the tazobactam-inhibited enzyme peptide elution profile at 230nm which differed between the tazobactam inhibited and uninhibited enzyme were collected and prepared for amino acid analysis as described in section 2.16. Amino acid analysis of the acid hydrolysate was carried out by Dr. J. Fox of Alta Bioscience, Department of Biochemistry, Birmingham University. The amino acid elution profile obtained is shown in Figure 23.



* plus unknown chemical compound(s) not composed of amino acids. Amino acids represented by one letter code

Figure 23. Amino acid elution profile for pooled HPLC fractions from the only region of the peptide elution profile (230nm) of the tryptic digest of tazobactam-inhibited *P. aeruginosa* SAI^{const} β-lactamase which differed from the peptide elution profile (230nm) of the tryptic digest of the uninhibited *P. aeruginosa* SAI^{const} β-lactamase. Quantitative determination of amino acid composition was carried using ion exchange chromatography (LKB) with post-column ninhydrin derivitisation. The identity of each amino acid was then determined by comparison with authentic standards and estimated quantitatively by peak area. Fraction absorbance was measured at 570nm (all amino acids except proline) and 440nm (for proline). Amino acids with acidic or neutral polar side chains were eluted from the column before those with basic or non-polar side chains.

The amino acid composition of the pooled HPLC fractions from the separation of the tryptic digest of the tazobactam-inhibited *P. aeruginosa* SAI^{const} β-lactamase is shown in Table 11.

The results from analysis of the tryptic digest fragments of the inhibited enzyme suggest that the fragments EIGSVSK, GPLDAE and KTGS are present in the additional peak in the elution profile from the tazobactam-inhibited enzyme. The KTG, DAE and SxxK motifs are highly conserved amongst the chromosomal β-lactamases which would support their possible involvement in the active site of the enzyme.

Table 11. Amino analysis of pooled HPLC fractions from the separation of the tryptic digest of tazobactam inhibit *P. aeruginosa* SAI^{const} β-lactamase

Amino Acid		Concentration nM/Sample
Asp	D	2.5
Thr	T	1.0
Ser	S	6.2
Glu	E	3.9
Pro	P	0.91
Gly	G	9.2
Ala	A	2.5
Cys	C	0.08
Val	V	0.82
Met	M	0.41
Ile	I	0.89
Leu	L	0.31
Tyr	Y*	0.51
Phe	F	0.54
His	H*	4.08
Lys	K	0.32
Arg	R	-

* plus unknown chemical compound(s) not composed of amino acids. The results are calculated using an appropriate response factor.

Quantitative amino acid analysis of pooled HPLC fractions from the only region of the peptide elution profile (230nm) of the tryptic digest of tazobactam-inhibited *P. aeruginosa* SAI^{const} β-lactamase which differed from the peptide elution profile (230nm) of the tryptic digest of the uninhibited *P. aeruginosa* SAI^{const} β-lactamase.

Interestingly, Y and H were found in the analysis to be bound to an unknown molecule, which could be tazobactam or some fragment of the tazobactam molecule. In this case both tyrosine and histidine may be involved in the binding of tazobactam to the active site of the enzyme. There are relatively few histidines in the *P. aeruginosa* SAI^{const} β-lactamase sequence. Most of the histidine residues occur within a region near to the conserved sequences which may support the involvement of a histidine in inhibitor binding. The modelling studies carried out also suggested the involvement of a distant tyrosine residue in inhibitor-binding, and this could be the tyrosine identified in the amino acid analysis. Obviously it is important to include these amino acid sequence segments in the construction of the active site model.

Labelling by tazobactam showed that the reaction of the enzyme with the inhibitor had cross-linked a number of peptide fragments which are not normally linked. In fact the active site of the enzyme has been shown to be composed of a number of distant areas of the polypeptide chain brought together upon protein folding.

Further amino acid sequencing of the fragments needs to be carried out to confirm the sequence segments and the exact identity of the residues involved.

6.7 Discussion

The results show that all the enzymes conform to classical kinetics and showed progressive irreversible inhibition except for the reaction of *S. aureus* PC1 and *E. cloacae* β-lactamases with tazobactam. In the case of *S. aureus* PC1 β-lactamase the reaction of tazobactam appeared to be progressive but reversible. This was in agreement with the findings of Cullmann (1990) who investigated the reaction kinetics of a number of enzymes with tazobactam and came to similar conclusions for the reaction of tazobactam with the *S. aureus* PC1 β-lactamase.

The results of section 6.3 showed: cloxacillin was a competitive inhibitor of *P. aeruginosa* SAI^{const}, *E. cloacae* P99 and *S. aureus* PC1 β-lactamases; ampicillin was a competitive inhibitor of *P. aeruginosa* SAI^{const} and *E. cloacae* P99 β-lactamases; cefotaxime was a competitive inhibitor of *P. aeruginosa* SAI^{const} and *E. cloacae* P99 β-lactamases; whilst tazobactam acted as a competitive inhibitor of *E. cloacae* P99 and *S. aureus* PC1 β-lactamases.

A competitive inhibitor often closely resembles in some respects the substrate whose reaction(s) it inhibits. Because of this structural similarity, the inhibitor and the substrate compete for the same binding site on the enzyme. When either the substrate molecules or inhibitor molecules bind, the other cannot. The enzyme bound inhibitor then either lacks the appropriate reactive group or is held in an unsuitable position with respect to the catalytic site of the enzyme or to other potential substrates, for a reaction to take place. Either the inhibitor reacts to form a complex which does not lead to product formation, or the inhibitor may be the product of the reaction and bind to the same enzyme form as the substrate. In either case the inhibitor must dissociate from the enzyme and be replaced by a molecule of substrate before a reaction can take place at

that particular enzyme molecule.

The effect of a competitive inhibitor is to decrease the apparent affinity of the substrate for the enzyme without any effect on the reactivity of the enzyme-substrate complex once formed (no effect on V_{max}). A competitive inhibitor decreases the apparent value of the specificity constant and increases K_m.

The kinetic results suggested that ampicillin, cloxacillin and cefotaxime, as competitive inhibitors, must bind directly with the residues of the active site of the β-lactamase. In the case of *E. cloacae* P99 and *S. aureus* PC1 β-lactamases the tazobactam molecules must also interact with the same residues in the active site as the substrate. This suggests that in any modelling studies these inhibitors should be docked into the active site of the enzyme according to the Knowles model.

However, in the case of the *P. aeruginosa* SAI^{const} β-lactamase, the results of the plots of 1/V against E_0 showed that tazobactam was an irreversible inhibitor of this enzyme. As an irreversible inhibitor tazobactam should be docked into the active site of the enzyme in the modelling studies. Further support for this mode of docking comes from the inhibition kinetics observed when tazobactam is not pre-incubated with *P. aeruginosa* SAI^{const} β-lactamase. Without pre-incubation tazobactam acted as a competitive inhibitor of the enzyme so suggesting that it binds directly to the active site.

Irreversible inhibitors are useful in the investigation of the active site of an enzyme, since the inhibitor, unlike the substrate, will remain firmly bound to one of the amino-acids of the enzyme and thus act as a marker to enable it to be identified. The irreversible binding of tazobactam to the active site of the *P. aeruginosa* SAI^{const} β-lactamase was used to identify the residues of the active site involved in the reaction by peptide fragment analysis experiments and molecular modelling studies described in chapter 7. The amino acid analysis suggested the presence of a number of residues which may form part of the important conserved sequence motifs EIGSVSK, GPLDAE and KTG as well as a implicating Y and H residues in the tazobactam binding reaction.

There are several reports in the literature concerning the conformational flexibility of the class C and class A β-lactamases. The reaction of *S. aureus* PC1 β-lactamase with tazobactam is biphasic and progressive which may be consistent with a substrate-induced deactivation process involving reversible conformational changes like

that observed with the substrate induced deactivation of other penicillinases (Kiener and Waley, 1977). Recently the carbapenem antibiotic, imipenem, has been shown to induce reversible conformational changes in β-lactamase I which are associated with transient inhibition (Monk and Waley, 1988). Kinetic behaviour involving the formation and decay of transiently-inhibited enzyme complexes as well as biphasic progressive irreversible inactivation can also occur through conformational changes of the enzyme. Transient inhibition observed with tazobactam and *S. aureus* PC1 β-lactamase could be due to a less active enzyme conformation, induced by turnover of tazobactam. The rate of relaxation of this conformation to the active native state may be slower than the rate of decay of the inhibitor enzyme complex. Such behaviour has been implied from the study of the conformation response of penicillinase induced by β-lactamase A-type substrates (Zyk and Citri, 1967).

The biphasic reaction kinetics observed with *S. aureus* PC1 β-lactamase and tazobactam may be due to a two step binding process where two discrete steps are involved in the binding of the inhibitor to the enzyme. Perhaps there is an initial slow binding step rate determining step followed by rapid equilibrium. The inhibitor might bind tightly at first and then convert to a more strongly bound intermediate such as the change between a trigonal intermediate to a tetrahedral one. Perhaps an inhibitor binds, then causes a conformational change in the protein before a secondary binding reaction occurs.

Further studies need to be conducted to see whether tazobactam is capable of inducing major changes in the conformation of β-lactamases. Firstly, do high concentrations of tazobactam cause precipitation of the β-lactamase without significant turnover of the inhibitor? Secondly, does irreversibly inactivated enzyme tend to precipitate, especially during dialysis? Precipitation of β-lactamase when exposed to high inhibitor concentrations may reflect secondary binding of the inhibitor to non-specific sites exposed by extensive conformational changes.

Several reports are consistent with the suggestion that transient inhibition results from a substantial conformational change in the enzyme at the acyl-enzyme stage. β-Lactamase, transiently inhibited by β-lactams such as cloxacillin or methicillin, become sensitive to the effects of iodine (Brenner and Knowles, 1981), ammonium sulphate (Pain and Virden, 1979) and guanidinium chloride (Kiener *et al.*, 1980) than the native

enzyme. Hydrogen exchange of the polypeptide amide hydrogens is significantly altered in the presence of cloxacillin. Reaction with antibodies or chemical cross-linking of the native enzyme can prevent the substrate deactivation phenomenon (Pain and Virden, 1979; Carrey *et al.*, 1984). Some of these observations can be interpreted to indicate a substantial change in the mobility or dynamics of the protein, rather than a conformational change. More direct evidence for the involvement of a conformational change comes from preliminary results on the stabilised, isolated inactivated complex formed from cloxacillin and β-lactamase I from *B. cereus*.

The importance of conformational change in both the active site and gross tertiary structure of β-lactamases following interaction with substrates or inhibitors is often neglected. Future investigations which concentrate on the conformational changes induced by inhibitors may reveal the more subtle aspects of the normal catalytic behaviour of β-lactamases.

It remains to be seen whether tazobactam will be a clinically useful inhibitor for the *S. aureus* β-lactamase because of the unusual reaction kinetics displayed by this enzyme with this particular inhibitor. The reversible nature of the inhibition may cause problems in clinical use, although it could be argued, if tazobactam is used in combination with a second β-lactam, that the initial period of inhibition before the recovery of enzyme activity may be sufficient to allow the β-lactam antibiotic to reach its target an kill the cell before the β-lactamase activity is restored.

CHAPTER 7

7 DETERMINATION OF THE CRYSTAL STRUCTURE OF TAZOBACTAM

7.1 Aims.

The aims of this section of work were:

(1) to determine the crystal structure of tazobactam and

(2) to determine the optimum molecular conformation of tazobactam for use in the docking studies.

7.2 Crystal structure of tazobactam

The crystal structure of tazobactam was determined as described in section 2.17. Tazobactam crystallised from a 70:30 ethanol/water solution as colourless hexagonal prisms which were found to exhibit orthorhombic symmetry, space group $P2_12_12_1$. The unit cell parameters (Table 12) were refined by least squares analysis of setting angles of 25 reflections obtained on an Enraf-Nonius CAD4 diffractometer. For collection of intensity data a crystal 0.83 mm long x 0.33 mm wide x 0.40 mm thick was selected. Although a ψ scan exhibited intensity variation of less than $\pm$ 6% from the mean, an empirical absorption correction was made with DIFABS (Walker and Stuart, 1983). By the ω - 2θ scan technique a total of 2464 observed reflections with $I >= 3\sigma$ were collected in the range $2 = <\theta =< 25°$ for graphite-monochromated MoKα radiation.

The structure was solved by direct methods using the RANTAN procedure within MULTAN (Main *et al.*, 1980) and was found to contain two independent molecules of tazobactam which have been distinguished as primed and unprimed. Missing non-hydrogen atoms were located in an electron density map followed by a least-squares refinement carried out with SHELX (Sheldrick, 1976). All hydrogen atoms except those in the carboxyl groups were introduced in calculated positions determined by the molecular geometry, methyl groups were treated as rotatable rigid bodies, and other H atoms were assumed to ride on their attached atoms. Carboxyl hydrogen atoms H(12) was located unambiguously in a difference electron density map.

Table 12. Summary of crystal data for tazobactam

Formula	$C_{10}H_{12}N_4O_5S$	system	orthorhombic
Mr	300.304	space group	$P2_12_12_1$
a, Å	10.230 (2)	Z	8
b, Å	14.396 (2)	wavelength	0.71069 Å
c, Å	17.291 (2)	μ	0.23 mm^{-1}
V, $Å^3$	2546.5	D_x	1.57 gcm^{-3}
reflections collected	2849		
index limits 0<=h<=12, 17<=k<=2, 0<=1<=20; parameters refined 394;			
extinction parameter* 0.000518; unique data (I>3σ) 2464; R, ω_R 0.033, 0.045			

* the value of X in the correction factor [1-0.0001 X F^2/sinθ] to be applied to the calculated structure factor.

Two plausible alternatives were found for H(12'); but one was selected because it gave the molecule a heat of formation from MOPAC (Stewart, 1989) 20 kcal mol^{-1} lower than the other, its intramolecular geometry was better, and only it engaged in reasonable hydrogen bonding. Coordinates and isotropic temperature factors were refined for H(12) and H(12') in the final cycles, along with isotropic temperature factors for other H atoms, coordinates and anisotropic thermal parameters for non-hydrogen atoms, and an empirical extinction correction. The observed reflections were weighted by $1/[\sigma^2(F) + 0.000519\ F^2]$. At termination no parameter shifted by more than 0.03 e.s.d.,the final discrepancy index was R= 0.033, and no peak in a difference electron density map exceeded 0.30 e $Å^{-3}$. The structures of the two tazobactam molecules are shown as ORTEP drawings (Johnson, 1976) with their numbering schemes in Figure 24.

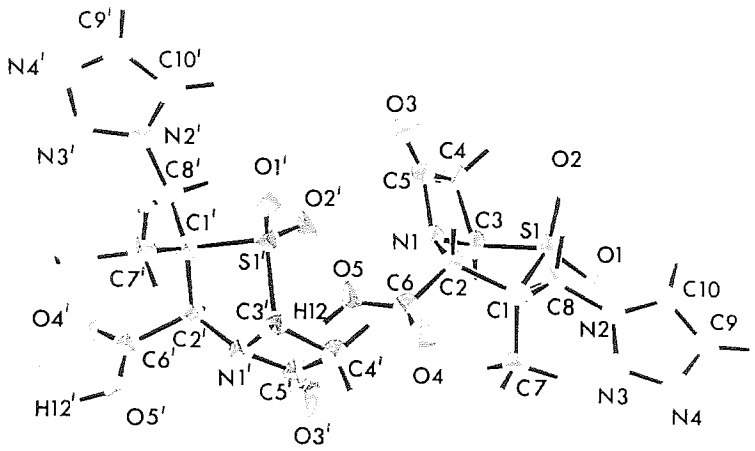


Figure 24. Computer generated ORTEP plot of the unprimed and primed crystal structures of tazobactam.

The principal differences between the primed and unprimed molecules are observed in the positions of the triazolylmethyl and carboxylic acid moieties, and the main torsion angle differences between the two molecules are given in Table 13.

Table 13. Conformationally significant torsion angles and principal differences between the primed and unprimed crystal structures of tazobactam

Primed		Unprimed	
Bond	Angle	Bond	Angle
1. S(1)-C(1)-C(8)-N(2)	-77.0(3)°	S(1')-C(1')-C(8')-N(2')	-87.8(3)°
2. C(1)-C(8)-N(2)-N(3)	-100.1(3)°	C(1')-C(8')-N(2')-N(3')	-100.8(4)°
3. C(1)-C(2)-C(6)-O(4)	39.9(4)°	C(1')-C(2')-C(6')-O(4')	78.8(4)°
4. C(1)-C(2)-C(6)-O(5)	-144.1(3)°	C(1')-C(2')-C(6')-O95')	-98.2(4)°

The important bonds in the unprimed and primed molecules of tazobactam with their appropriate torsion angles.

A significant difference between the torsion angles of bonds 1, 3 and 4 was noted. The thiazolidine rings are puckered into an envelope conformation, with S(1) and S(1') out of the plane of the other four atoms by 0.813(1)Å and 0.818(1)Å respectively, both with asymmetry parameters 7 DCs of 1.3°. The alternative penicillin conformation with S(1) in plane and the β-carbon C(2) out of plane would have positioned the sulphone oxygen atoms symmetrically. Nevertheless, the S(1) envelope conformation appears preferable since this is also observed in sulbactam (Brenner and Knowles, 1981) (DCs = 6.1°). This same thiazolidine conformation appears in X-ray and NMR studies on penicillin β-sulphoxides, where DCs = 5.3° for penamicillin (Labischinski *et al.*, 1987) and DCs = 2.5 ° for cloxacillin derivatives. It has been suggested (Labischinski *et al.*, 1987) that this geometry is inimical towards binding to the target transpeptidase enzyme; however, this may aid in binding to β-lactamases.

An interesting feature apparent from the crystallographic study was the presence of intermolecular hydrogen bonding between the C-2 carboxylic acid proton and and the N-4 triazolyl nitrogen. The crystal structure unit cell of tazobactam is shown in Figure 25.

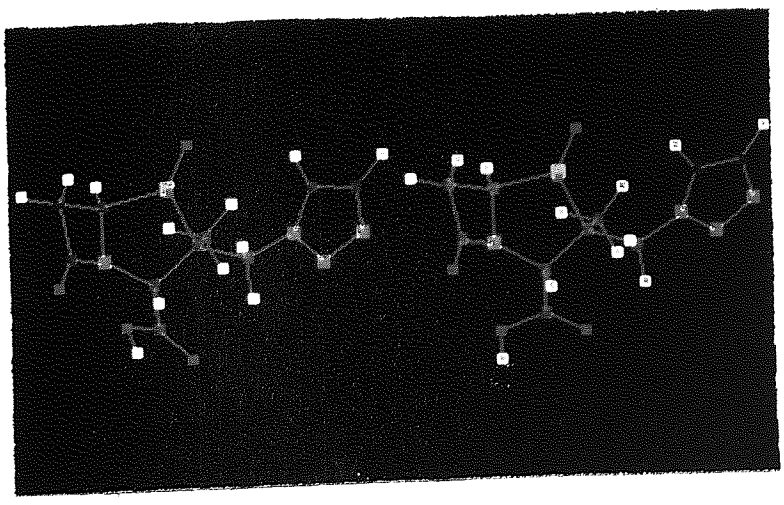


Figure 25. Crystal structure unit cell of tazobactam. Unit cell of tazobactam displayed in Chem-X format. Unit cell contains both primed and unprimed molecules. Careful examination of the figure reveals the differences in position of the triazolylmethyl and carboxylic acid groups between the primed and unprimed molecules. The relative position of each molecule is such that an intermolecular hydrogen bond could be formed between the C-2 carboxylic acid proton and the N-4 triazolyl nitrogen.

Examination of the Cambridge Crystallographic database for compounds containing both triazole and carboxylic acid functionalities indicated literature precedent for this type of hydrogen bonding (Chapius *et al.*, 1977). Table 14 shows the appropriate hydrogen bond lengths and angles.

Table 14. Hydrogen bond data for the primed and unprimed structures of tazobactam

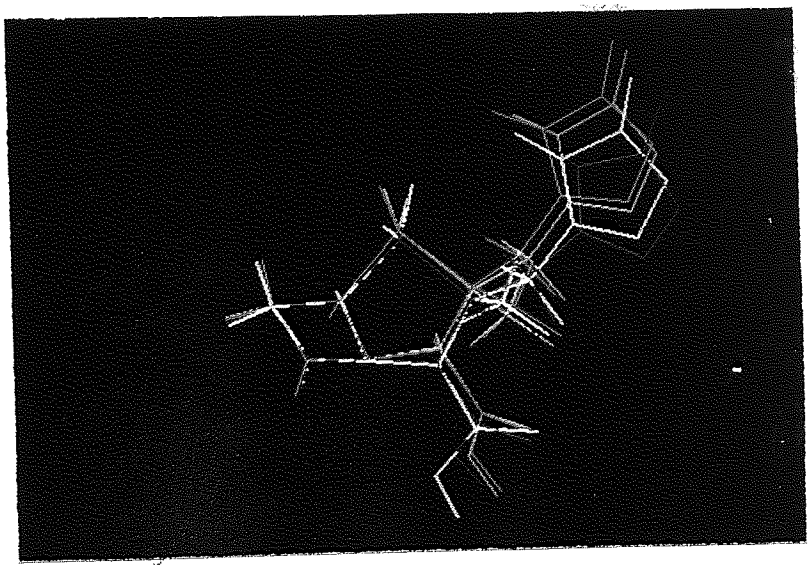
Bond	Donor-Acceptor Distance	Donor-H-Acceptor Angle
O(5)-H(12)-N(4)	2.667(4) Å	170(6)°
O(5')-H(12')-N(4')	2.682(5) Å	170(6)°

The optimum geometries of both independent molecules of tazobactam in the crystal structure were determined using MOPAC (Stewart, 1989), employing the MNDO/PM3 Hamiltonian. The independent structures optimised to virtually identical low energy conformations, giving a final heat of formation of -76.8 kcal mol^{-1} for the unprimed structure, and -76.5 kcal mol^{-1} for the primed structure. Before optimisation the corresponding figures were -38.0 and -32.8 kcal mol^{-1}. The thiazolidine ring was less puckered after optimisation than in the crystal, and as a result, the triazole ring occupied a slightly different region of space. The C(1)-S(1)-C(3)-C(4) torsion angles of 131.0(2)° and 130.1(3)° for the unprimed and primed molecules decreased to 123.5° and 119.4 upon optimisation.

Figure 26 shows the minimised and unminimised primed and unprimed structures of tazobactam superimposed by their β-lactam rings. The figure shows that minimisation causes a shift in the positions of the atoms in the crystal structure of tazobactam. The main differences between the primed and unprimed molecules are observed in the positions of the triazolylmethyl and carboxylic acid moieties, and the main torsion angles (Table 14). The main difference between the minimised and unminimised structures are that the thiazolidine ring was less puckered after optimisation

than in the crystal, and as a result, the triazole ring occupied a slightly different region of space. The torsion angles for the unprimed and primed molecules also changed slightly upon optimisation.

a



b

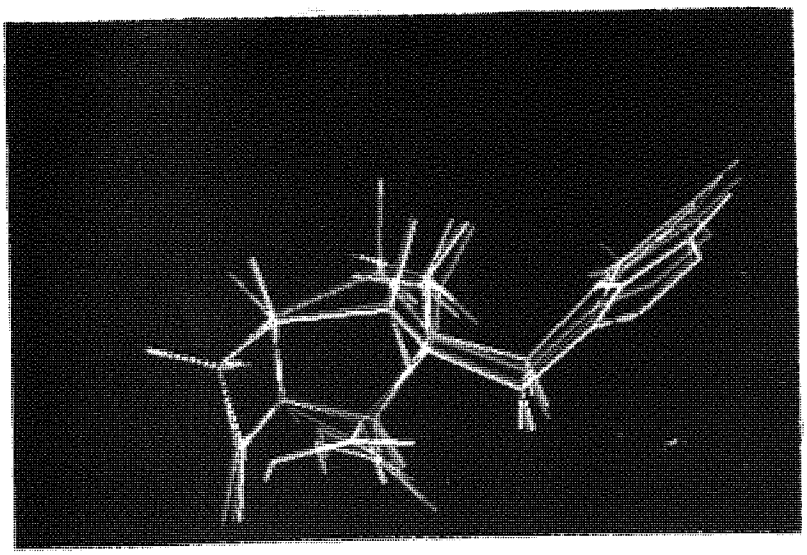


Figure 26. MOPAC minimised and unminimised primed and unprimed crystal structures of tazobactam MOPAC minimised and unminimised primed and unprimed crystal structures of tazobactam displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Individual molecules have been superimposed by β-lactam ring atoms. Molecules are colour coded as follows; minimised primed = red, minimised unprimed = green, unminimised primed = blue, unminimised unprimed = yellow. Figure 26b shows Figure 26a rotated around 180°.

The MOPAC calculations also suggested why tazobactam exhibits intermolecular hydrogen bonding between the C(4) triazole nitrogen and the H(12) acid proton as described above. An electrostatic potential map of the optimised unprimed crystal structure of tazobactam (Figure 27) show that the areas of most negative potential (dark blue) are centred around the triazole ring nitrogens, and the carboxyl oxygen atoms have a slightly less-negative potential.

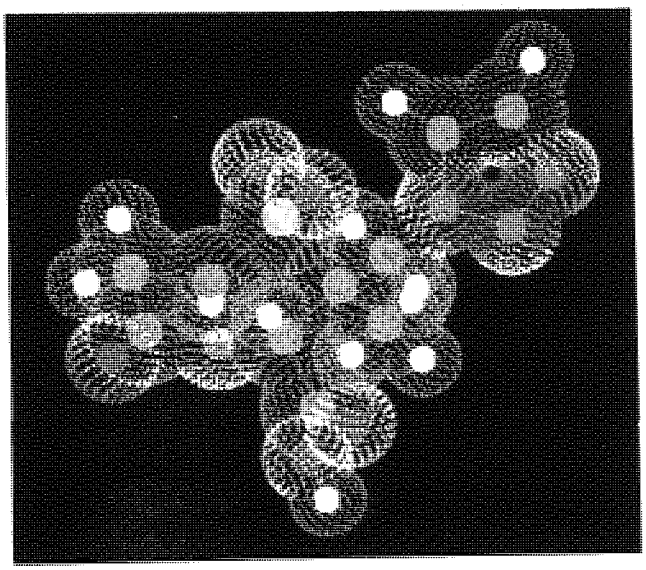
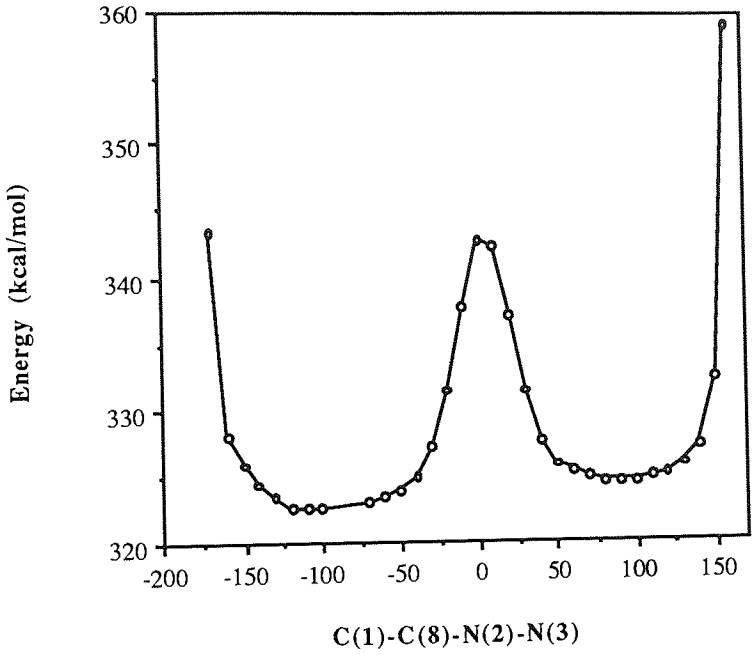


Figure 27. Electrostatic potential map of the optimised unprimed crystal structure of tazobactam. Electrostatic potential of the unprimed crystal structure of tazobactam after MOPAC optimisation. The dot surface has been colour coded as follows: dark blue <-55; light blue -55 to -45; white -45 to -25; orange >-25 kcal/mol.

The conformational flexibility of both independent molecules was investigated using molecular mechanical calculations with the modified MM2 parameters (Allinger, 1977) available within Chem-X. Separate rotations around the C(1)-C(8) and C(8)-N(2) bonds at approximately 10° intervals followed by calculation of the molecular mechanics energy showed that, for the unprimed molecule, the energy barrier to rotation about either of these bonds is no greater than 50 kcal mol-1. In the case of the primed molecule slightly more energy is required to 'flip' the triazole ring through 180°. Figures 28a and b 28c and d show the plots of torsion angle against energy for rotation around the C(1)-C(8) and C(8)-N(2) bonds for both independent molecule.

a



b

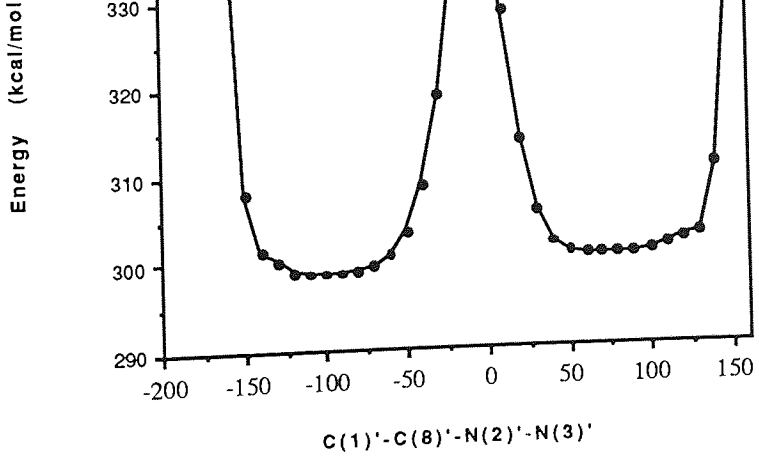
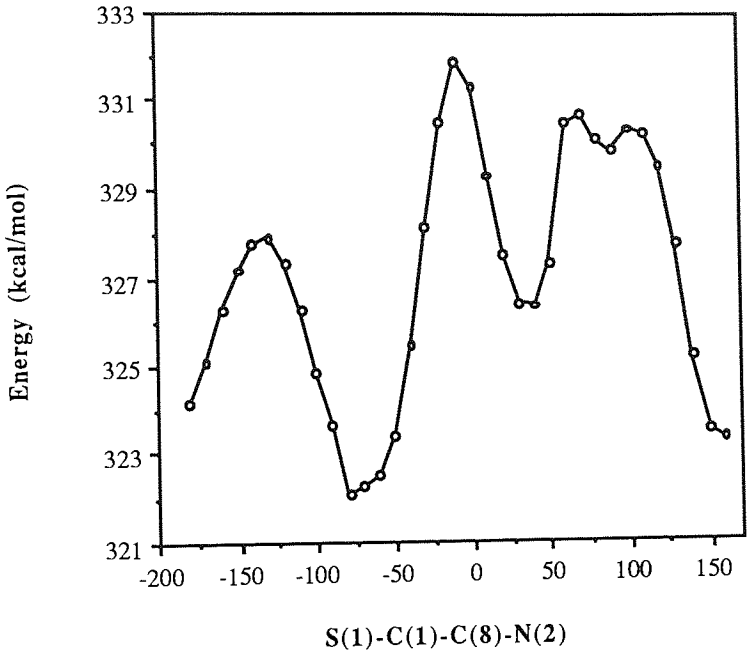


Figure 28 a and b. Plots of MM2 molecular mechanics energy (kcal/mol) against torsion angle for rotation about the C(1)-C(8)-N(2)-N(3) bond for the unprimed (o) and primed (•) molecules of tazobactam

c



d

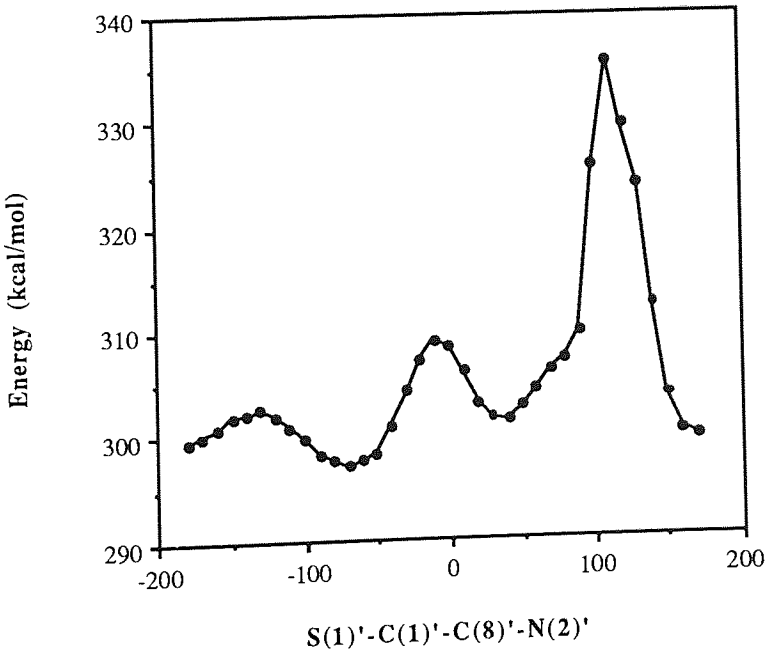


Figure 28 c and d. Plots of MM2 molecular mechanics energy (kcal/mol) against torsion angle for rotation about the S(1)-C(1)-C(8)-N(2) bond for the unprimed (o) and primed (•) molecules of tazobactam

The equivalent torsion angles for the molecules in the crystal structures were: -77.0° and -100.1° for the unprimed molecule and -87.8° and -100.8° for the primed molecule respectively. Figure 29 shows the equivalent energy contour plot for the unprimed molecule.

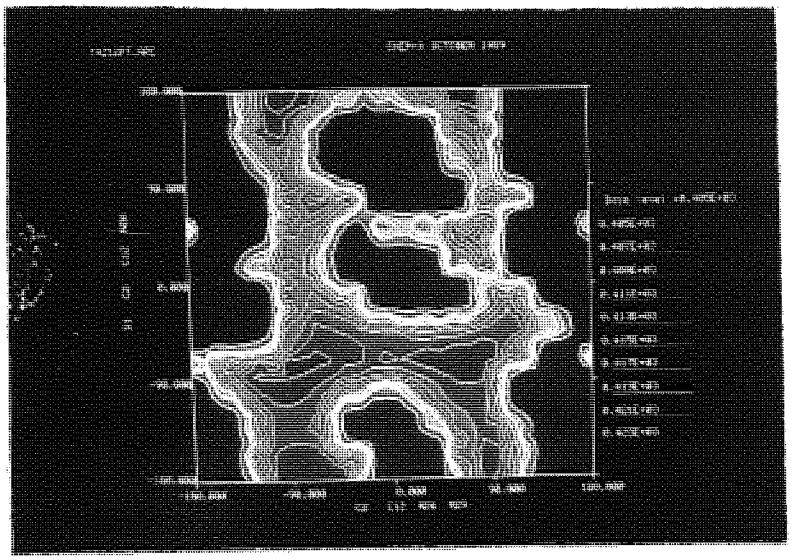


Figure 29. Energy contour plot for the unprimed molecule. Energy contour map of Chem-X molecular mechanics energy (Kcal/mol) for rotations around S(1)-C(1)-C(8)-N(2) (y axis) and C(1)-C(8)-N(2)-N(4) (x axis). Energy levels (kcal/mol) are colour coded as follows: blue, 405 (darkest shade) to 411(lightest shade); yellow, 419; orange 421; red 423.

It can be seen that the conformations of tazobactam with the triazole ring rotated through 180° occupy low energy troughs, and it is possible that the energy required to overcome the barrier to rotation could be supplied to the molecules when in solution.

The only other part of the molecule that is able to undergo free rotation is the C(2) carboxylic acid group. Conformational analysis of rotation around the C(2)-C(6) bond as described above indicated that the energy barrier to rotation is very low, with the difference between the lowest and the highest energy conformations of both molecules no greater than 9 kcal mol^{-1}. It is therefore reasonable to assume that when in solution, sufficient energy can be obtained to rotate the carboxylic acid group into the most advantageous conformation for binding to the enzyme.

The electrostatic isopotential surfaces of tazobactam and sulbactam (Purvis,

1991) were calculated after MOPAC minimisation using a CACheTM workstation (Tektronix). Figure 30 shows the electrostatic isopotential surface drawn over the MOPAC minimised structure of tazobactam. Figure 31 shows the electrostatic isopotential surface drawn over the MOPAC minimised structure of sulbactam.

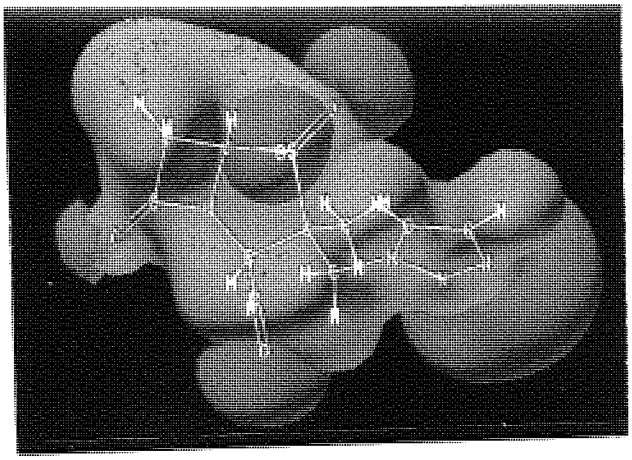


Figure 30. Electrostatic isopotential surface over the MOPAC minimised structure of tazobactam. The electrostatic isopotential surface of tazobactam was calculated after MOPAC minimisation using a CACheTM workstation (Tektronix). The +/-0.05 a.u. (+/-31.4 kcal mol^{-1} or +/-131.3 kJ mol^{-1}) surfaces are coded blue for negative and red for positive electrostatic potential.

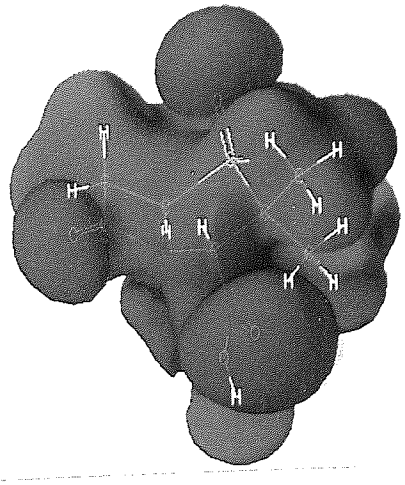


Figure 31. Electrostatic isopotential surface over the MOPAC minimised structure of sulbactam. The electrostatic isopotential surface of sulbactam was calculated after MOPAC minimisation using a CACheTM workstation (Tektronix). The +/-0.05 a.u. (+131.4 kcal mol^{-1} or +/-131.3 kJ mol^{-1}) surfaces are coded blue for negative and red for positive electrostatic potential.

A notable feature is the pronounced negative potential surrounding two of the three triazole group nitrogen atoms (N2 and N3) of tazobactam. Sulbactam lacks the triazole moiety, however, the electro-potential over the rest of the molecule's structure (β-lactam and thiazolidine ring) which is common to both tazobactam and sulbactam appears almost identical. Compared to the related inhibitor sulbactam, which lacks the triazole ring, crystalline tazobactam exhibits very similar β-lactam geometry and the same S(1) envelope conformation of the thiazolidine ring. However, in both independent molecules of tazobactam a triazole ring nitrogen atom accepts an intermolecular hydrogen bond; similar interactions by this moiety of tazobactam with a hydrogen bond donor on the enzyme, which are impossible for sulbactam or clavulanic acid, could account for its enhanced inhibitory power against some enzymes. Semi-empirical molecular orbital calculations show pronounced negative potential there. Molecular mechanics supports the hypothesis that the carboxyl group can rotate freely and the triazole ring can "flip".

The enhanced β-lactamase inhibitory activity (Gutmann *et al.*, 1986) of tazobactam compared to that of sulbactam or clavulanic acid is presumably due to the presence of the triazole function and thus it is possible that this ring must participate in some favourable interaction within the active site of the β-lactamase. The molecular basis of enzyme inhibition as postulated by Brenner and Knowles (1984a) involves acylation of an active site serine by the β-lactam, followed by a nucleophilic attack on the C(3) position of the β-lactam by another active site residue. In order to study these hypotheses, it was necessary to establish the most favourable conformation of the molecule which could be adopted.

To determine what effect, if any, the triazolylmethyl group exerts on the penam skeleton, the crystal structures of tazobactam were compared to that of the β-lactamase inhibitor sulbactam. Several parameters have been studied in an attempt to correlate the biological activity of β-lactams with structure. The chemical reactivity of the amide bond (Johnson *et al.* , 1949) and a suitable distance between the β-lactam oxygen and the carboxylic acid carbon (Cohen, 1983) (the Cohen distance) have both been used in structure activity relationships. The reactivity of the amide bond has been related to the C=0/C-N bond lengths and the pyramidality of the β-lactam nitrogen (Johnson *et al.*, 1949).

These parameters were determined from the crystal structures of sulbactam (Brenner and Knowles, 1981) and tazobactam and are summarised in Table 15.

Table 15. β-Lactam ring parameters for sulbactam and tazobactam *

	sulbactam	tazobactam unprimed	primed
Distance of β-lactam N from plane	0.377Å	0.418(4)Å	0.387(4)Å
C=O bond length	1.202Å	1.185(5)Å	1.188(6)Å
C-N bond length	1.388Å	1.410(5)Å	1.382(5)Å
Distance from β-lactam O to carboxyl C	3.904Å	4.021(4)Å	4.026(5)Å

* Estimated standard deviation in parentheses.

Table 15 shows some of the important bond lengths that are known to be important to the reactivity of the molecule as shown by structure activity relationship studies. Data are given for the primed and unprimed molecules of tazobactam and the related compound sulbactam.

It can be seen that values for the two independent molecules of tazobactam are very similar to those of sulbactam although the Cohen distance is at the upper limit of the range ascribed to active compounds (Chung and Chodosh, 1989) (3.0-3.9Å). The pyrimidality of the β-lactam nitrogen was expressed as the distance from this atom to the plane through C(2), C(3) and C(5). These figures indicate that the β-lactam N lies slightly further out of the plane of the 3 atoms surrounding it than the corresponding atom in sulbactam. Consequently, amide resonance is hindered and as has been postulated by Johnson *et al.*, (1949), the susceptibility of the β-lactam to nucleophilic attack should be slightly greater. It does not, therefore, appear that the triazolyl group exerts any major effect on the geometry of the β-lactam system of tazobactam (compared to sulbactam) indicating that its enhanced β-lactamase inhibitory activity does not stem

from any altered chemical reactivity. In general, the bond angles, bond distances and torsion angles for the two independent molecules of tazobactam bear a very close similarity to the analogous data for sulbactam. The principal differences were noted around the carboxylic acid functionality, with the C-OH bond length decreased from 1.327(6)Å in sulbactam to 1.288(4)Å and 1.306(5)Å in the unprimed and primed molecules respectively.

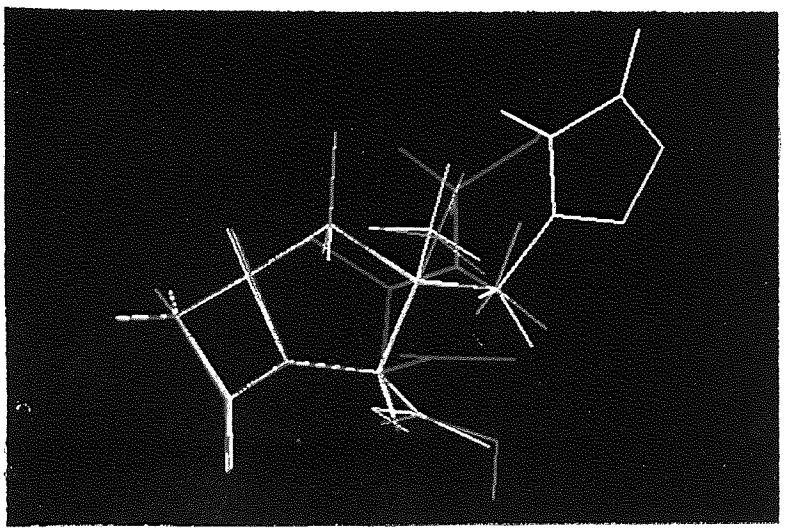


Figure 32. MOPAC minimised structures of tazobactam, sulbactam and clavulanic acid. Structures of tazobactam, sulbactam and clavulanic acid displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Individual molecules have been superimposed by β-lactam ring atoms. Molecules are colour coded as follows; tazobactam = green, sulbactam = blue, clavulanic acid = red

Careful examination of Figure 32 shows that the β-lactam rings of all three molecules are very closely similar although the β-lactam ring N of tazobactam appears to lie slightly out of plane formed by the other 3 atoms of the β-lactam ring of sulbactam and clavulanic acid. The thiazolidine rings of both tazobactam and sulbactam are superimposed, however, the thiazolidine ring of clavulanic acid lies in a different plane and is bent away from those of tazobactam and sulbactam. The other difference between sulbactam and tazobactam is the position of the carboxylic acid functionality. The length of the C-OH bond is slightly longer in sulbactam than it is in tazobactam. The greatest difference is the position of the various substituent groups, with the most obvious

differences between the three molecules being the triazole ring of tazobactam which neither sulbactam nor clavulanic acid have.

Analysis of the crystallographic data for sulbactam shows that an intermolecular hydrogen bond exists between the β-lactam carbonyl oxygen and the carboxylic acid proton. In the case of tazobactam, the enhanced electron density present on the triazolyl ring makes possible the hydrogen bonding described above. The possibility also exists for the formation of such a hydrogen bond between the triazole nitrogen and a suitable peptide residue within the enzyme active site.

CHAPTER 8

8 MOLECULAR MODELLING OF THE ACTIVE SITE OF THE CHROMOSOMAL β-LACTAMASE OF *P. AERUGINOSA* SAI^{const} AND ITS INTERACTIONS WITH INHIBITORS.

8.1 Aims.

The aims of this section were:

(1) to predict and model the tertiary structure of the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase and

(2) to propose a mechanism and the residues responsible for the binding and inhibition/ hydrolysis of β-lactams by the *P. aeruginosa* SAI^{const} chromosomal β-lactamase using this model and other biochemical and kinetic data obtained in this study.

8.2 Choice of template for the prediction of the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

The inhibition of DD-carboxypeptidases by β-lactams has long been known (Cooper, 1956; Blumberg and Strominger, 1974) to involve a covalent interaction. Since both DD-carboxypeptidases and β-lactamases react specifically and covalently with β-lactams, and because of their structural resemblance between their natural substrates, D-alanyl-D-alanine-terminating peptides and β-lactams, respectively, Tipper and Strominger (1965) suggested that β-lactamases were evolutionary descendants of DD-carboxypeptidases. There is now much evidence, both structural and functional, for this evolutionary relationship (Samraoui *et al.*, 1986). Although there is only limited evidence for homology on a primary level (Waxman and Strominger, 1983), it seems likely that there is much secondary and tertiary similarity (Kelly *et al.*, 1986; Samraoui *et al.*, 1986). It is also now clear that a number of elements of primary structure, adjacent to the active sites, have been conserved. These include, first, a serine residue (Ser 70) whose hydroxyl group is the primary active-site nucleophile during turnover of β-lactams by β-lactamases and inhibition of DD-carboxypeptidases by β-lactams (Ser 90, *P. aeruginosa*, Ser 62, *S. aureus*). Also apparently important functional groups are the

ammonium ions of Lys 73 (Lys 93, *P. aeruginosa*, Lys 65, *S. aureus*) and Lys 237 (Thr 246, *P. aeruginosa*, Ala 300, *S. aureus*)(Lys 237 may be replaced by a histidine residue) and, perhaps, the carboxyl group of Glu 166 (Asp 245, *P. aeruginosa*, Glu 225, *S. aureus*). The catalytic role of these functional groups has been recently discussed (Herzberg and Moult, 1987; Moews *et al.*, 1990; Ellerby *et al.*, 1990; Knap and Pratt, 1991). In terms of size both the DD-carboxypeptidases and the class C β-lactamases are very similar.

Functionally, there is further overlap between the two types of active sites. Both DD-carboxypeptidases (Nguyen-Disteche *et al.*, 1986) and β-lactamases catalyse the hydrolysis and aminolysis, by specific amino acid and peptide amines of substrates with the same general structure.

The essential difference between the two groups of enzymes, which must have been crucial factors in the evolutionary process producing a β-lactamase from a DD-carboxypeptidase is: β-lactamases have the ability to catalyse hydrolysis of acyl-enzymes formed on reaction with β-lactam antibiotics. It has been suggested (Govardhan and Pratt, 1987; Herzberg and Moult, 1987) that this development involved the appearance in β-lactamases of a specific and possibly substrate-occluded binding site for water. β-Lactamases do not have the ability to catalyse the hydrolysis and aminolysis of acyclic amides (peptides). This would likely be an important feature of β-lactamase evolution since the interference in cell-wall biosynthesis created by peptide-hydrolysing β-lactamases could be very serious.

The use of a serine residue in the catalytic mechanism, the close relationship of the substrate molecule to a peptide, and the probable evolutionary relationship of the class A β-lactamases to cell wall peptidases all suggest that there are parallels with the well-studied serine proteases of the chymotrypsin and subtilisin families. These two structural classes of serine proteases have very similar, although apparently evolutionary independent, catalytic mechanisms (Robertus *et al.*, 1972; Kraut, 1977) which have some functional similarity to the mechanism thought to be used by the β-lactamases. Each serine protease has an active site serine, an adjacent histidine residue, and a buried aspartic acid interacting with the histidine. Two NH groups form hydrogen bonds to the carbonyl oxygen adjacent to the scissile bond of a substrate, creating an environment known as an oxyanion hole (Kraut, 1977) like the one reported to exist within β-

lactamases. In addition, a serine residue in the subtilisin family lies at the amino terminus of a buried α-helix, suggesting a role for the helix dipole in catalysis (Hols, 1985) which also occurs in the β-lactamases.

There is very little primary structure homology between the serine proteases and the β-lactamases but there is a degree of homology between the elements of the active site. These include a serine residue, whose hydroxyl group is the primary active-site nucleophile and an oxyanion hole (Kraut, 1977; Marquart *et al.*, 1983). A comparison of the active site of the serine protease- protease B from *Streptomyces griseus* and the class A β-lactamase of *S. aureus* PC1 (the α and β- carbon atoms of the active site serine residue, Ser 62 in the β-lactamase and Ser 195 in protease B) and the main chain nitrogens of the respective oxyanion holes shows a high degree of superimposition. The root mean square discrepancy between the four atoms in the two structures after superimposition is 0.3Å.

An important feature of the serine protease structure is the oxyanion hole which is formed by Gly 193, Ser 195, Asp 194 and Asn 155. The two aligned structures indicate that the ammonium group of Lys 65 in the*S. aureus* β-lactamase fall close to the side chain of the catalytic His 57 in protease B. There is no analogous residue to Asp 102 of protease B in the β-lactamase but Glu 225 (Asp 245 in *P. aeruginosa* chromosomal β-lactamase) interacts with Lys 65 (Lys 93, *P. aeruginosa*) (Herzberg and Moult, 1987; Moews *et al.*, 1990; Baguley, 1990), and is shielded from the solvent in the presence of a substrate. The alignment also results in a similar spatial orientation of the respective substrates, with the equivalence of the scissile bond and its neighbouring atoms closer than 1Å.

In the class C *P. aeruginosa* enzyme the predicted position of the phenolic oxygen atom of tyrosine 230 lies approximately 0.5Å from the N2 of the essential histidine in trypsin 57. This indicates that Tyr 230, as its anion, acts as a general base during catalysis of β-lactam hydrolysis in a way similar to that of His 57 in trypsin (Marquart *et al.*, 1983). Tyr 230 of *P. aeruginosa* could be homologous to Tyr 159 in the R61 DD-carboxypeptidase and Ser 160 of the *S. aureus* PC1 class A β-lactamase (a conserved residue) the spatial arrangement of the hydroxyl groups and the conserved lysines are also equivalent.

There have also been reports of the serine proteases- human leukocyte elastase (Doherty *et al.*, 1986, 1990; Finke *et al.*, 1990) and porcine elastase being inhibited by a β-lactam compound (Navia *et al.*, 1987), however, not all β-lactams are capable of inhibiting serine proteases or being used by them as substrates which suggests that there is some crucial difference between the proteins.

The final candidate for the model is the class A β-lactamase of *S. aureus* PC1. There is much evidence for the functional similarity between both the class A and class C β-lactamases (Jaurin and Grundstrom, 1981; Knott-Hunziker *et al.*, 1979; Cohen and Pratt 1980; Fisher *et al.*, 1980; Knott-Hunziker *et al.*, 1982; Joris *et al.*, 1984). A comparison of the primary structure (amino acid sequences) of the two groups of β-lactamase show that there is very little sequence homology, except in the region of the active site. Comparison of the sequence of the β-lactamase of *S. aureus* PC1 and those of a number of class C β-lactamases shows that both have the SxSK motif which contains the reactive serine, the KxG which contains a lysine residue which may be important in the binding of β-lactams and the GxxD motif, which contains an important aspartic acid residue as well as a number of single conserved residues; a tyrosine, a lysine. In terms of size, the class A β-lactamases are smaller than class C β-lactamases.

Functionally, the mechanisms by which both groups of enzymes work must be identical is borne out by the fact that a number of elements of primary structure, adjacent to the active sites, have been conserved. These include, first, a serine residue (Ser 70) (Ser 90, *P. aeruginosa*, Ser 62, *S. aureus*) whose hydroxyl group is the primary active-site nucleophile during turnover of β-lactams and several other important functional groups; the ammonium ions of Lys 73 (Lys 73, *P. aeruginosa*, Lys 65, *S. aureus*) and Lys 237 (Lys 342, *P. aeruginosa*, Lys 298, *S. aureus*), and the carboxyl group of Glu 166 (Asp 245, *P. aeruginosa*, Glu 225, *S. aureus*). However the differences in substrate and inhibitor profiles indicates that there are some important differences between the two groups of enzymes. These differences are most likely the residues which are involved in the binding or recognition of substrates by the enzyme.

A comparison of the atoms of the active site serine and conserved lysine, two residues away and the α-carbon coordinates of the peptide backbone of the threonine and serine residues in the β-lactamase of *S. aureus* PC1 and the valine and threonine in the *Streptomyces* R61 DD-carboxypeptidase show that the structures superimpose with

a total root-mean square discrepancy of 0.02Å.

There are many complete crystal structures available for members of the serine protease group of enzymes and their recognition, binding and catalytic mechanisms have been well studied and documented. Their significance for use as a model is that they are thought to act by a mechanism similar to that of the β-lactamases. However, this mechanism is not identical and other differences in substrate and inhibitor profiles led to their rejection for use in this study.

The α-carbon crystal structure of the *Streptomyces* R61 DD-carboxypeptidase has been available for some time and has been closely studied (Kelly *et al.*, 1985). Its size and sequence homology and related catalytic mechanism made it a suitable candidate for the active site model, however, an error in the α-carbon backbone tracing of this enzyme in the area close to the active site excluded it from use as a possible model.

The complete crystal structure of the class A β-lactamase of *S. aureus* PC1 β-lactamase (Hertzberg and Moult, 1987) became available from the Brookhaven Protein Databank during the course of the study (PDB update 1991). In view of its functional relatedness to the chromosomal β-lactamases and the structural similarity of the active site elements with those of *Streptomyces* R61 DD-carboxypeptidase and trypsin it was decided to use this structure as a basis for the active site model of *P. aeruginosa* SAI^{const} β-lactamase

8.3 Identification of active site residues for the model of *P. aeruginosa* SAI^{const} chromosomal β-lactamase.

One major limitation of the available modelling techniques is the number of atoms which can be minimised in the CHARMm molecular modelling package. This limited the modelling study to the active site of the *P. aeruginosa* SAI^{const} β-lactamase.

An initial examination of the α-carbon coordinate crystal structure of *S. aureus* PC1 β-lactamase was conducted to determine which sections of the protein backbone were within binding distance of the active site serine. Figure 33 shows the α-carbon crystal structure of the *S. aureus* PC1 β-lactamase.

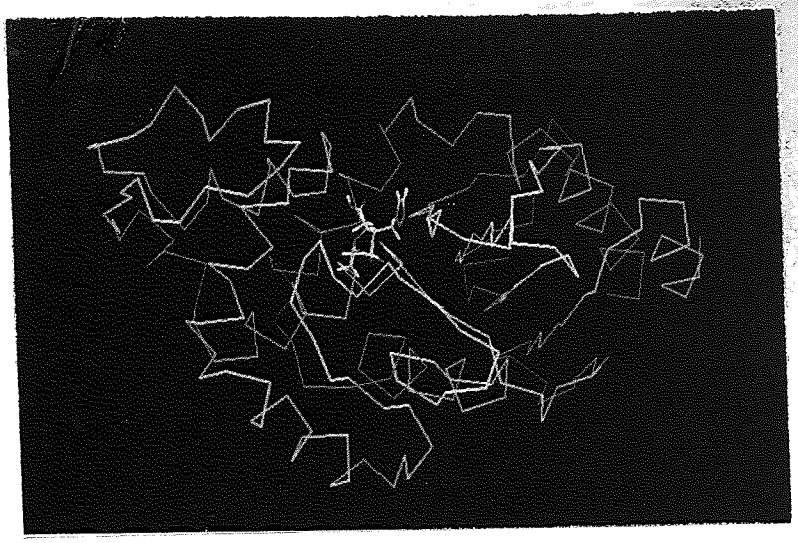


Figure 33. α-Carbon crystal structure of the *S. aureus* PC1 β-lactamase. α-Carbon crystal structure of *S. aureus* PC1 β-lactamase displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. α-Carbon coordinates have been joined using a special bonding algorithm available in QUANTA. The active site serine is coloured red.

Examination of Figure 33 shows that the protein has a compact spheroid shape with a central groove which contains the active site with dimensions of approximately 4.5 nm x 4.85 nm x 5.8 nm. Four sections of the polypeptide chain make up the active site. These are residues GKEVKFNSDKRFAYASTSKAINSAILLEQV (residues 49-75), which contains the important SxxK (residues 62-65) motif of the active site, KALIEASMTYS (residues 143-160), TNPVRYEIELNY (residues 220-231), which includes a conserved glutamic acid residue, and KVADKSGQAITYASR (residues 294-306), which includes the important KxG triad (residues 298-300). Sequence alignments also show that another important conserved structural motif is SDN (residues 160-164) which occur in a loop. Several other important architectural features are also apparent which may be important for the interaction of this protein with its substrates. The first and most important is the positioning of the reactive serine at the N-terminus of an α-helix This helical configuration results in a dipole moment on the helix with a positive partial charge at the amino end of the helix which can activate the serine residue making it more reactive than any of the other serines in the enzyme. Glu 225 is also positioned at the amino-terminus of an α-helix which again will be influenced by a helix dipole moment and perhaps helps in the activation of this residue in order for it to react and

form a salt bridge with Lys 65. The conserved KxG residue is at the start of a β-sheet opposite the α-helix which contains the conserved SxxK motif. Tyr 231 and Lys 237 are positioned on a Ω-loop which lines the side of the narrow opening linking the external solvent and the interior of the enzyme. Perhaps the importance of this loop is in the guidance of the the substrate into the active site or assists in the formation of the oxyanion hole with the residue Glu 225.

To select the corresponding active site segments from the *P. aeruginosa* β-lactamase amino acid sequence, the sequences of the *S. aureus* PC1 β-lactamase (Herzberg and Moult, 1987), the *P. aeruginosa* enzyme (Lodge *et al.*, 1990), and other class C β-lactamases were aligned (results not shown). An alignment of the amino acid sequence segments though to be involved in the active sites of these enzymes is shown in Table 16.

Table 16 shows the residues in the *P. aeruginosa* β-lactamase which correspond to the residues identified in the α-carbon crystal structure of *S. aureus* PC1 as being important or involved in the active site are: segment 1, RRVTPETLFEIGSVSKTFTATLAG (residues 78-101), which contains the important reactive serine in the SxxK motif (residues 90-93); segment 2, TYAPGSQRLYS (residues 168-178), which contains the equivalence of the important SDN (residues 178-180) triad a conserved element in the class A β-lactamases; segment 3, GPGPLDAEGYGV (residues 240-251), which contains the conserved DAE triad (residues 245-247); and segment 4, RLLNKTGSTNGF (residues 338-349), which contains the conserved KTG triad (residues 342-344).

Comparison of the amino acid sequences shows several important points;

(1) that there is a high degree of homology between the amino acid sequences of the *P. aeruginosa*, *E. coli*, *E. cloacae* P99 and *C. freundii* class C β-lactamases with identical amino acids occurring at the same positions in each of the sequences examined, suggesting close structural and functional relationships between the enzymes of this group. The sequence homology with the *S. aureus* PC1 class A enzyme is much lower, perhaps showing a difference in its evolutionary origin.

(2) a number of conserved structural motifs occur in the sections of the class C β-lactamase identified as making up the active site. Of particular interest are the LFExSx

SKTF (residues 85-95), DA-E (residues 245-247), and KTG (residues 342-344) motif. These elements are conserved in the active sites of most penicillin-recognising enzymes. Studies by a number of authors confirm that many of these conserved residues may be involved in the binding and catalytic mechanism of the enzyme.

(3) there are also regions of the amino acid sequences where, although the individual amino acids are not identical or conserved, the amino acid sequences still have identical chemical properties, owing to the substitution of a chemically similar residue.

(4) where the sequence does differ, the residues may be responsible for the differences in substrate and inhibitor profile of the enzymes, influencing the recognition and binding of potential substrates by the enzyme. This is perhaps best illustrated by a comparison of the class A and class C enzymes. These enzymes are known to have very different inhibitor and substrate profiles (even within the same enzyme class differences in substrate and inhibitor profiles are observed) which could be explained by the differences in amino acid sequences (compare the serine protease group of enzymes where the shape of the active site is determined by a few key variant residues and hence the particular substrate the enzyme can utilise). Experiments have shown that the chemical modification or mutation of certain residues altered the substrate and inhibitor profiles of the *Strep. albus* G β-lactamase.

8.4 2-Dimensional modelling of the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

The structure of the *S. aureus* PC1 β-lactamase shows that the residues identified as being important in the enzyme active site occur in well-defined structural elements that may have a profound effect on their reactivity and in creating the active site. To determine whether the residues identified in the *P. aeruginosa* SAI^{const} active site occurred in similar secondary structure elements a structural propensity prediction was carried out. The results of the prediction are shown in Figure 34.

		74 104
Segment 1	*P. aeruginosa*	KEDGRRVT-PET-LFEIGSVSKTFTATLAG-YAL
		46 76
	E. coli	IAKKQPVT-QQT-LFELGSVSKTFTGVLGG-DAI
		68 98
	E. cloacae	IAANKPVT-PQT-LFELGSISKTFTGVLGG-DAI
		46 76
	C. freundii	IANNHPVT-QQT-LFELGSVSKTFNGVLGG-DAI
		46 • • • 76
	S. aureus PC1	TKSGKEVKFNSDKRFAYASTSKAINSAILLEQVP
		143 183
Segment 2	*P. aeruginosa*	RQWQPTYAPGSQRLYSNPSIG
		145 165
	E. coli	QNWQPAWAPGTQRLYANSSIG
		157 177
	E. cloacae	QNWQPQWKPGTTRLYANASIG
		145 165
	C. freundii	QNWQPQWTPGAKRLYANSSIG
		145 165
	S. aureus PC1	L------IE-ASMTYS--DNT
		236 254
Segment 3	*P. aeruginosa*	PLRVGP-GPL-D-AE--------GYGVKTS
		•••••220 243
	E. coli	AVHVSP-GAL-D-AE--------AYGVKST
		228 246
	E. cloacae	AVRVSP-GML-D-AQ--------AYGVKTN
		•••••220 243
	C. freundii	PVHVSP-GQL-D-AE--------AYGVKSS
		220• 243
	S. aureus PC1	-----TNPVRYEI-ELNYYSP--KSKKDTS
		334 352
Segment 4	*P. aeruginosa*	LEGQRLLNKTGST-N-GFGAY
		•••293 308
	E. coli	AVRASWVHKTGAT-G-GFGSY
		327 345
	E. cloacae	PVKASWVHKTGST-G-GFGSY
		•••293 308
	C. freundii	AVKASWVHKTGST-G-GFGSY
		293 308
	S. aureus PC1	---YKVADKSGQAITYASRND

Table 16. Comparison of the amino acid sequence segments involved in the active site of the β-lactamases of *P. aeruginosa* , *E. coli*, *E. cloacae* P99, *C freundii* and *S. aureus* PC1.Amino acid sequence segments were aligned manually. The alignment included the class C chromosomal β-lactamases of *P. aeruginosa*, (Lodge *et al.*, 1990), *E. coli,* (Jaurin and Grundstrom, 1981), *E. cloacae* P99, (Joris *et al.*, 1984), *C. freundii,* (Lindberg and Normark, 1986a) and the class A β-lactamase of *S. aureus* PC1, (Herzberg and Moult, 1987). The sequence numbering for *E. coli, C. freundii,* and *S. aureus* PC1 β-lactamases corresponds to that used by Joris *et al.* (1988). The sequence numbering for *P. aeruginosa* and *E. cloacae* P99 β-lactamases corresponds to that used by Lodge *et al.* (1990). Deletions in the sequence are denoted by -. Unnumbered residues are denoted by •. The amino acid segments were used to construct the active site of the chromosomal β-lactamase of *P. aeruginosa*

The predication showed that the enzyme is mostly an α + β protein: that is, it is made up of mostly α-helices and β-sheets. There are 12 α-helices (boxed in yellow) and 9 antiparallel β-sheets (boxed in blue) arranged as α-helix, β-sheet, β-sheet, α-helix, α-helix, α-helix, α-helix, α-helix, α-helix, α-helix, α-helix, β-sheet, β-sheet, β-sheet, α-helix, β-sheet, β-sheet, β-sheet, β-sheet, α-helix, the elements are linked by β-turns.

The active site serine (Ser 90) occurs at the N-terminus of one of the helices in the all-helical region. The regular, repeating pattern of residues in a helical configuration may produce a dipole moment on the helix with a positive partial charge at the amino end of the helix. This sets up a delocalised positive field gradient which may play a role in initial attraction of β-lactams to the active site region of the enzyme. In order to be active against bacteria an antibiotic must have a carboxylate group in close proximity to the β-lactam nitrogen. Also, the position of the catalytic serine at the N-terminus of the helix may activate this residue making it more reactive than the other serines in the enzyme.

The positioning of the reactive serine at the end of a helix also may explain the constant spacing observed between this residue and the lysine that occurs in all the known penicillin-recognising enzyme sequences. The pattern, with no known variations in any of the serine β-lactamases, is SxxK. The periodicity of an α-helix is 3.6 residues per turn of helix. If this lysine is to participate in binding or catalysis, or both, as it is highly conserved nature suggests, then it must be spaced two residues away from the serine to traverse the first turn of helix, bringing the basic side chain back into the active site.

The conserved glutamic acid residue (Glu 247) of the DAE triad occurs at the N-terminus an α-helix. The helical configuration may produce a dipole moment on the helix with a positive partial charge at the amino end of the helix. This sets up a delocalised positive field gradient which may play a role in initial attraction of Lys 93 from the SxxK motif and help in the establishment of the salt bridge known to exist between these two residues in the *S. aureus* PC1 β-lactamase. Also, the position of the residue at the N-terminus of the helix may activate this residue making it more reactive than the other glutamic acids in the enzyme. Usually there is another acidic residue two residues downstream which may have some influence on the properties of residue Asp 245.

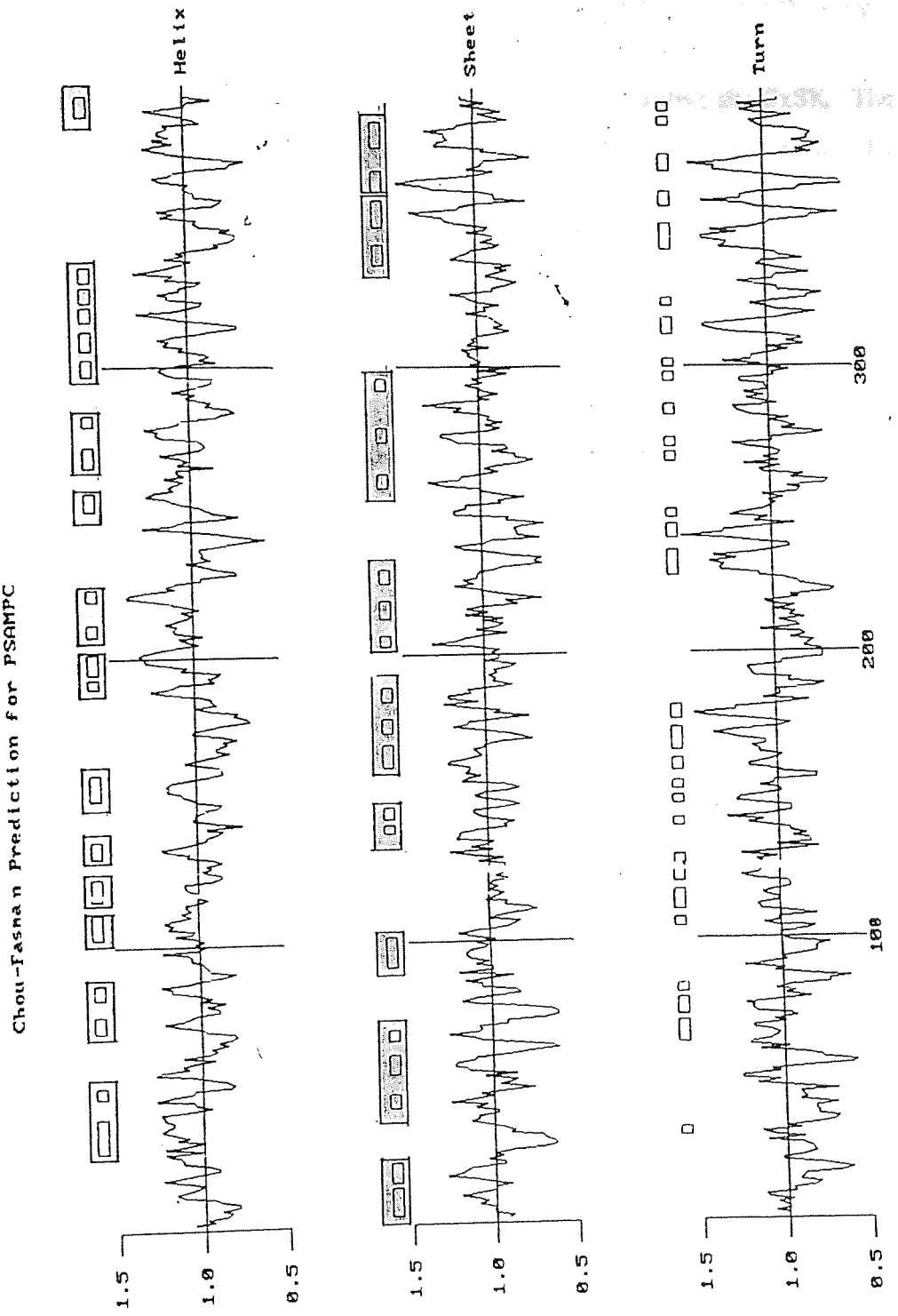


Figure 34. Chou-Fasman graphical secondary structure prediction for the amino acid sequence of *P. aeruginosa* chromosomal β-lactamase. Two dimensional structure for the chromosomal β-lactamase of *P. aeruginosa* was predicted using the Chou-Fasman option from the PROTYLSE 2-D structure prediction package. 2-Dimensional structure is represented as α-helix, β-turn and β-sheet. Separate propensity graphs are plotted for each structural element. Graphs show the propensity value for individual amino acids to form any of the structural types plotted against number of amino acid. Propensity values for amino acids range from +0.5 = strong propensity to form structure to -0.5 = strong propensity not to form structure.

The KTG triad occurs in a β-sheet region adjacent to the active site SxSK. The conserved lysine may be influenced by the chemical environment created by surrounding residues which is identical to the chemical environment of the KSG triad in the *S. aureus* PC1 β-lactamase. This favours the formation of hydrogen bonds with the lysine residue which may be important in helping bring about the second nucleophilic attack on the β-lactam

The propensity prediction shows that the likely secondary structure formed by the amino acid sequence of the chromosomal β-lactamase of *P. aeruginosa* does resemble that of the class A β-lactamase of *S. aureus* PC1. The conserved amino acids occur in the identical type of secondary structure element. This information was used in the modelling of a 3-Dimensional structure for the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}

8.5 3-Dimensional modelling of the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

The active site model of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} was constructed by the methods described in section 2.18.2. The data from previous sections 8.2-8.4 showed that several important features which had to be taken into consideration when constructing the 3-Dimensional model:

(1) Certain amino acid sequence segments must be included in the model as these contain conserved structural motifs or their equivalents that were found to be involved in the active site of *S. aureus* PC1 β-lactamase. These are: segment 1, RRVTPETLFEIGSVSKTFTATLAG (residues 78-101), which contains the important reactive serine in the SxxK motif (residues 90-93), segment 2, TYAPGSQRLYS (168-178), which contains the equivalent of the important SDN triad (residues 177-179) a conserved element in the class A β-lactamases, segment 3, GPGPLDAEGYGV (residues 240-251), which contains the conserved DAE triad (residues 245-247), and segment 4, RLLNKTGSTNGF (residues 338-349), which contains the conserved KTG triad (residues 342-344).

(2) The SxxK (90-93), YxN (178-180), DAE (245-247) and KTG (342-344) amino acid sequences occur in a particular type of tertiary structure: the active site serine

(Ser 90) occurs at the N-terminus of an α-helix. The conserved glutamic acid residue (Glu 247) of the DAE triad occurs at the N-terminus of an α-helix. A salt bridge exists between Lys 93 from the SxxK motif and Glu 247. The KTG triad occurs in a β-sheet region adjacent to the active site SxSK.

(3) Amino acid analysis of the tryptic digest fragments of tazobactam inhibited *P. aeruginosa* SAI^{const} β-lactamase suggested that the amino acid sequence fragments EIGSVSKTF (residues 87-95), GPLDAE (242-247) and KTGS (residues 342-345) were directly involved in the active site of the enzyme. Tyrosine and histidine residues were also found in the fragments suggesting that these residues were also involved in the binding of tazobactam to the β-lactamase active site. The tyrosine may come from the conserved YAN sequence which is functionally and sterically identical to the SxN motif of the class A β-lactamases (Mottl *et al.*,1991).

The active site model was constructed using the amino acid sequence segments identified in (1) and (3) with the conserved S, E and KTG being incorporated into the secondary structure elements identified in (2). The resulting active site model is shown in Figure 35.

The figure shows that the resulting 3-dimensional model corresponds well with the results obtained from the 2-dimensional predictions. All the important amino acid motifs occur within the type of 3-dimensional structure predicted in the 2-dimensional modelling.

This model was then used for docking studies with tazobactam, clavulanic acid and sulbactam to help to identify the residues involved in substrate binding and to propose a mechanism by which β-lactam hydrolysis may occur.

8.6 Dynamic simulations on the active site fragments of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

One important point to remember when modelling any system is that the protein itself is not a static structure. Its component atoms are constantly vibrating. Most studies show the position of atoms at only one point in time. A more correct way of representing structure might be to use an average of the all the data sets of atom coordinates generated during the course of dynamic simulations. The degree of

movement possible by the peptide chain and the residue side chains may be an important

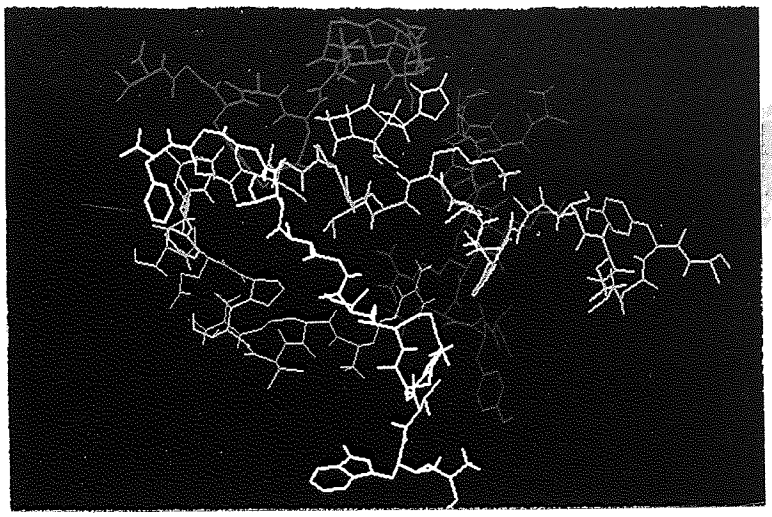


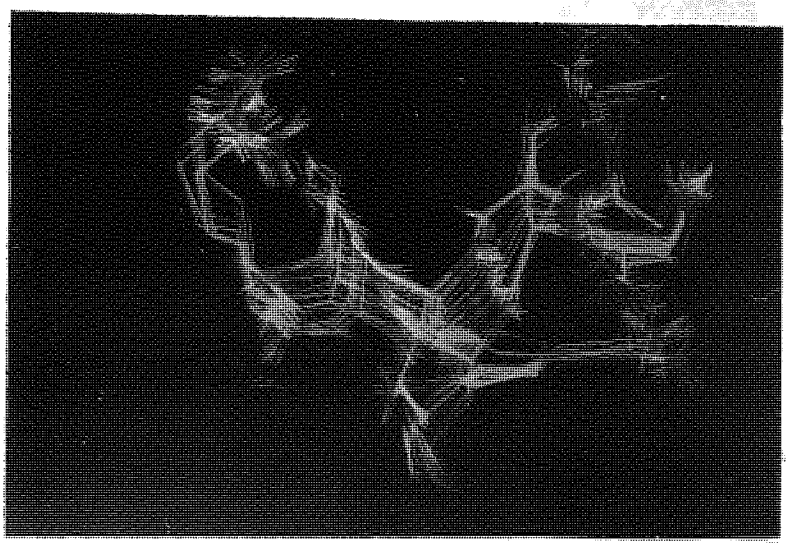
Figure 35. The CHARMm minimised predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. CHARMm minimised structure of the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Active site segments have been positioned relative to one another as they would be positioned in the actual crystal structure of the *S. aureus* PC1 β-lactamase. Items are colour coded as follows; active site segment 1 = yellow, active site segment 2 = red, active site segment 3 = blue, active site segment 4 = white.

feature of the interaction of the β-lactamase with β-lactams. The energy for this movement is supplied mainly from solution (solvation energy) with some coming from the heating effect. In order to determine the degree of movement possible for each of the residue side chains, dynamic simulations were carried out which would simulate the natural movement of the polypeptide chain in solution.

Figure 36 shows the results from the dynamics simulations carried out on each of the segments composing the enzymes active site. Each figure corresponds to 40 different conformations of the polypeptide chains which may be formed during motion of the protein.

Figure 36.

a



b

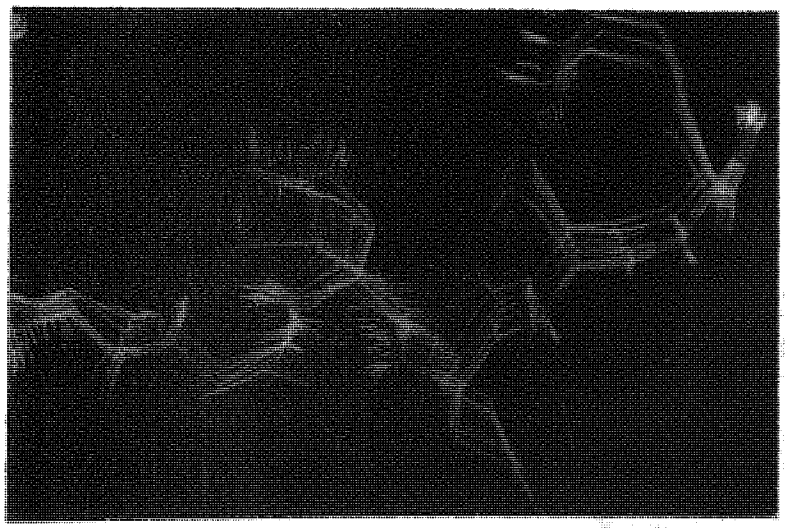
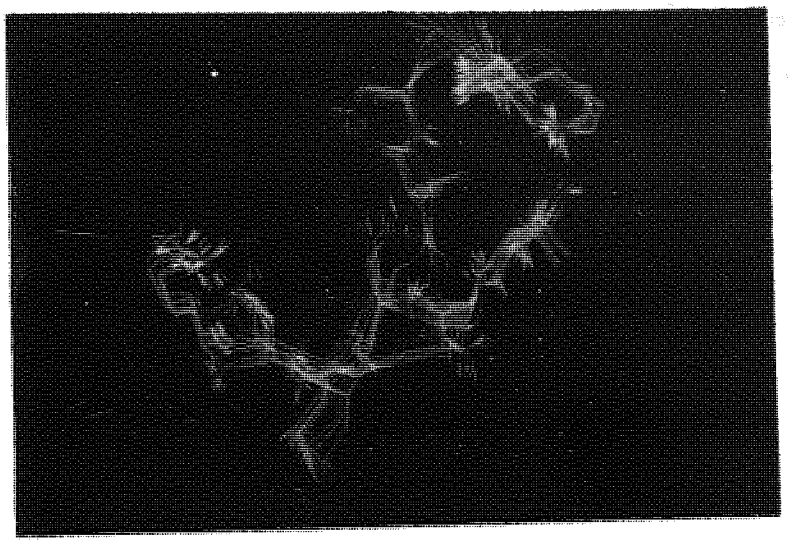


Figure 36 continued.

c



d

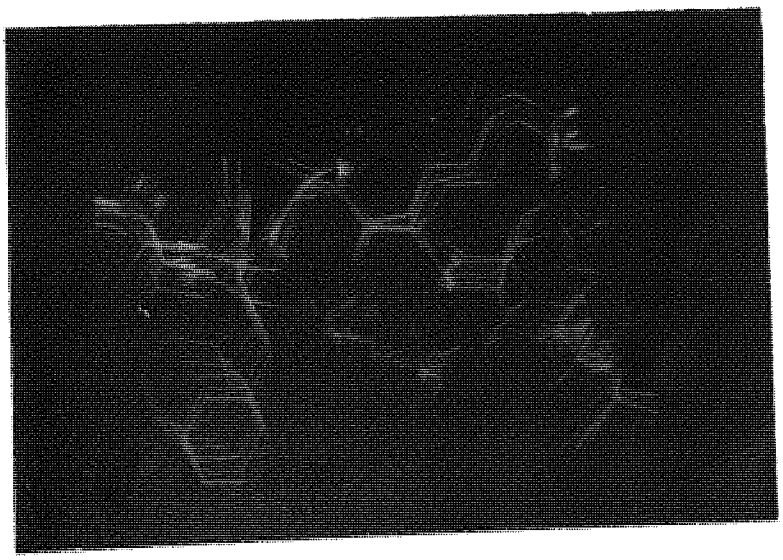


Figure 36. Dynamic simulations of active site segments of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.Dynamic simulations were carried out on each of the four segments which are thought to compose the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. Each of the MOPAC minimised segments of the active site under went dynamic simulations form the CHARMm molecular mechanics package. Bonds lengths were constrained using CHARMm constraints. Molecular system was heated to 300K. 40 sets of atom coordinate data were obtained and displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Figure shows data sets superimposed. Figure a = active site segment 1, b = active site segment 2, c = active site segment 3 and d= active site segment 4. Atoms are colour coded as follows; carbon = green, oxygen = red, nitrogen = blue, hydrogen = white.

The dynamic simulations of active site segment 1 shows that the side chain of Ser 90 is capable of rotation around the C1-C3 bond of approximately 60° along the plane of the peptide bond and 40° across the plane of the peptide chain which means that it is capable of moving into the active site cleft and also towards Lys 93. The side chain of Lys 93 also shows an unusual degree of movement (approximately 50° in the peptide plane and 60° across the peptide plane) towards Ser 90 (the positioning of this side chain may be one of the draw-backs of this type of minimisation in that a commonly reported error in minimisation is the attraction of free lysine side chains to the peptide backbone). This movement may be important in the positioning of Ser 90 to facilitate a nucleophilic attack on the β-lactam ring and in the case of Lys 93 to aid the possible formation of a salt bridge with Glu 247 which may be important for β-lactam hydrolysis, increase the possibility of hydrogen bonds being formed with the β-lactam which may help to position the substrate ready for further attack, or merely to assist in substrate binding. Ser 92 also shows a significant degree of movement (approximately 30° along the peptidé plane and 40° across the peptide plane) which might be sufficient to position it such that it could participate in the second nucleophilic attack and the formation of a second covalent linkage at C5 of the β-lactam. The changes in the positions of other atoms in the polypeptide fragment do not, at this time, appear to be significant.

The dynamic simulations carried out on active site segment 2 show that the whole polypeptide fragment has a significant degree of flexibility. Tyr 177 can move 90° about the plane of the polypeptide. Possibly this may be important as Tyr 177 may form an anion which then acts as a general base during β-lactam hydrolysis in the same way as the active site His 57 does in the serine proteases. It may also interact with the lysine residues from other fragments of the active site which might help to maintain its anionic charge in solution.

Segment 3 contains a number of highly conserved residues, in particular the PxGDAE residues. The dynamic simulations carried out on active site segment 3 show that residue Glu 247 has a high degree of flexibility being capable of rotating 180° across the plane of the polypeptide chain and 60° along the plane of the polypeptide chain. This degree of flexibility may be important for the formation of a salt bridge interaction with Lys 93 where it may be involved in the deacylation reaction, perhaps by

enhancing the nucleophilic attack of the water molecule. It is thought to be involved in an oxyanion hole type structure. The remaining residues do not exhibit a great deal of movement (e.g.Leu 243 19°, Asp 245 12°).

The dynamic simulations carried out on active site segment 4 show that the side chains of residues Phe 349 is capable of 120° rotation across the plane of the polypeptide chain. This perhaps is important in substrate positioning. The serine residue 4 residues away at position 345 is also capable of a limited degree of movement (30°) along the plane of the polypeptide chain. The only other residues which show a significant degree of movement are the two lysine residues (Leu 339 and Leu 340) which are able to rotate approximately 30° across the plane of the polypeptide chain and 30° along the polypeptide chain into the active site, and the arginine (Arg 338) side chain which is also able to move approximately 30° across the plane of the polypeptide chain in towards the active site and could be involved in substrate positioning or possibly in the case of asparagine form a hydrogen bond with the carbonyl oxygen of the β-lactam. This segment contains a number of conserved residues which are common to all penicillin-recognising enzymes and which are thought to be important in the hydrolysis of β-lactams. Ser 345 point out towards the surface but during the dynamic simulation it is capable of movement which may favour hydrogen bond formation through its main chain NH groups with those of Ser 90 to the carbonyl oxygen of the newly formed acyl-group after the initial nucleophilic attack has taken place to open up the β-lactam and aid in the formation of a structure very akin to the oxyanion hole seen in the serine proteases. The carbonyl oxygen of Ser 345 might also form a hydrogen bond with the acyl-amino NH of tazobactam which may then cause a conformational change in the broken β-lactam which might possibly be a rotation around the C3-C4 and N1-C4 bonds. The movement of the side chain of Lys 342 (30° across plane of polypeptide chain) and its neighbouring threonine (343) of the KTG box may aid its involvement in a charge-charge or more likely a hydrogen bond interaction with the carboxylate group on the thiazole ring of β-lactams which could be important in the binding and or positioning of tazobactam in the active site.

The component residues of the active site show a high degree of flexibility which may be very important in the orientation, binding and reaction of the substrate with the enzyme. The most important point is that the movement of any of these residues may

put them in a favourable position to interact or bond with the substrate or other residues which could be missed in static binding studies.

8.7 The importance of flexibility in the β-lactam in the reaction of the β-lactam with the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

In the same way that the polypeptide chain is not a static structure, the inhibitor molecule itself should not be thought of as an an absolutely inflexible molecule. As shown in section 7.2 the substituent groups of tazobactam are themselves capable of movement, the energy for which is thought to be supplied by solution. The other possible source of flexibility of the molecule is what happens on hydrolysis. The breaking of the β-lactam ring leads to a conformational change which may cause a significant repositioning of some parts of the molecule which may be important for the hydrolysis reaction or possibly the difficulty of hydrolysing certain compounds.

Studies on the DD-carboxypeptidase of *Streptomyces* R61 binding to inhibitors (Fisher *et al.*, 1980) show that there is no significant conformational change in the gross structure of the protein. The most significant changes observed were those in the actual shape of the inhibitor, especially the side chain constituents.

After the initial nucleophilic attack on the C3 position in the β-lactam ring, the molecule is capable of rotation which in turn puts strain on the C5 bond which as well as helping to position this bond so that a second nucleophilic attack can occur, may also increase the reactivity of this bond.

The triazole group of tazobactam has been shown to be able to flip, however, in the confines of the active site the side chain may become a fairly rigid body which may contribute to its resistance to hydrolysis.

8.8 Interaction of tazobactam with the active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}.

The results from chapter 6 show that tazobactam is an irreversible inhibitor of *P. aeruginosa* SAI^{const} β-lactamase. A plot of V_m against E_o shows that irreversible inhibition may be taking place and is indicative of an irreversible inhibitor which covalently binds to the active site of the enzyme. Kinetic studies where there was no

pre-incubation time of the enzyme with tazobactam showed that tazobactam behaved as a competitive inhibitor. Therefore it makes sense to dock the molecule into the active site of the enzyme.

The MOPAC minimised structure of tazobactam was docked into the predicted active site 8. The results of the docking studies are shown in Figures 37 to 40.

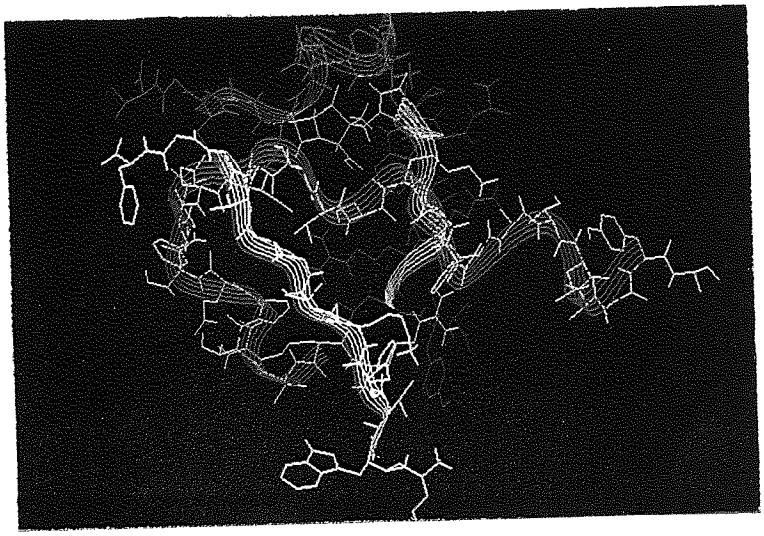


Figure 37. Tazobactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. MOPAC minimised structure of tazobactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Secondary structure is shown by ribbons which follow the folding of the peptide backbone . Items are colour coded as follows; tazobactam = green, active site segment 1 = yellow, active site segment 2 = red, active site segment 3 = blue, active site segment 4 = white.

Figure 37 shows the 4 polypeptide segments which are thought to compose the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase. Careful examination of the figure shows that the catalytic site is situated between several α-helices and the inner edge of a β-sheet. Next to this helix is a β-strand which also contains several of the important conserved amino acids and against which the β-lactam molecule (tazobactam) lies. These three segments of the enzyme are especially important: the 78-101 helix containing the reactive serine 90, the 338-349 β-strand and the 240-251 helix at the bottom of the binding site. The β-strand contains a tripeptide sequence (Lys 342,

Thr 343, Gly 344) which occurs in equivalent forms in all serine β-lactamases. These β-strand residues, while probably not catalytically functional, are possibly important for orientation of the antibiotic prior to attack by the Ser 90. The helical peptide segment at the bottom of the active site contains an invariant glutamic acid 245 which may assist deacylation by directing a water molecule to the acyl serine bond.

The lysine group (Lys 93) and the neighbouring threonine (Thr 94) group, are likely to be involved in charge-charge or hydrogen bond interaction with the requisite carboxylate group on the thiazole ring of β-lactams. The inhibitor may use an antiparallel hydrogen bonding with this β-strand in order to align itself for nucleophilic attack by Ser 90 on the adjacent helix. The serine's position at the N-terminus of the helix macrodipole will tend to lower the pKa of the hydroxyl proton, making it more reactive than usual for a serine hydroxyl proton. This proton lability may be assisted by a positively charged lysine, whose amino group lies within approximately 3Å of the hydroxyl group.

Figure 38 shows the 4 polypeptide segments which are thought to compose the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase superimposed on to the α-carbon coordinates of the class A β-lactamase of *S. aureus* PC1 which was used as the template for the *P. aeruginosa* SAI^{const} β-lactamase active site. Careful examination of the figure shows that the catalytic site is situated in one of two crevices on the enzymes surface (outlined by the α-carbon atoms). The crevice lies between several α-helices and the inner edge of a region of β-sheets. Viewed side-on the crevice appears as a depression in the protein surface measured as approximately 20Å long and about 5.5Å deep using on-screen geometry facility in QUANTA. The character of the amino acids composing the active site make it more hydrophilic than that of the related *Streptomyces* R61 DD-carboxypeptidase. Many of the amino acids making up this active site are conserved amongst the sequences of the chromosomal β-lactamases (Table 16).

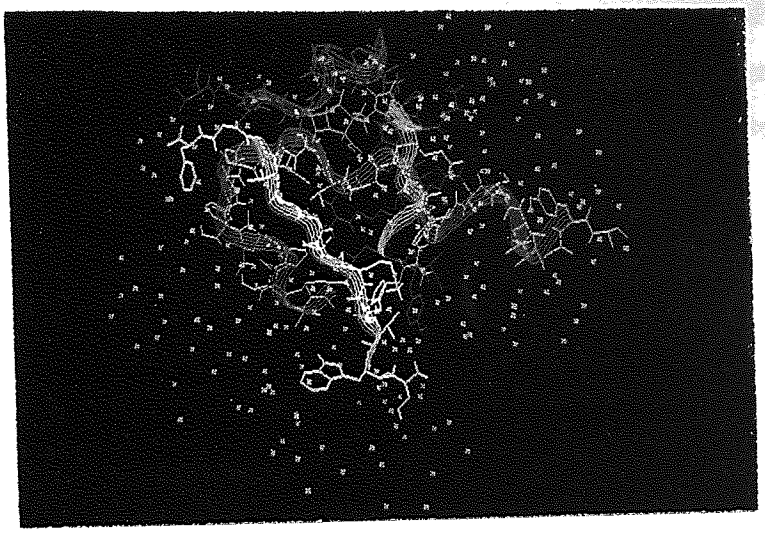


Figure 38. Tazobactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} superimposed on the α-carbon coordinates of *S. aureus* PC1 class A β-lactamase. MOPAC minimised structure of tazobactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} superimposed on the α-carbon coordinates of the class A β-lactamase of *S. aureus* PC1 displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Secondary structure is shown by ribbons which follow the folding of the peptide backbone. Items are colour coded as follows; tazobactam = green, active site segment 1 = yellow, active site segment 2 = red, active site segment 3 = blue, active site segment 4 = white, α-carbon atoms = yellow.

Figure 38 shows the 4 polypeptide segments which are thought to compose the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase. Careful examination of the figure shows that towards the edge of the molecule, the depression narrows into a gully which is lined by the side chains of Ser 116, Glu 117 and Thr 345, Asn 347. The side chain of Ser 90 lies in the floor of the depression, and the ammonium group of Lys 93, another conserved residue, is immediately adjacent to it. The side chain of the conserved Glu 247 also lies on the floor, where it may be capable of forming a salt bridge interaction with the side chain of Lys 93. On the opposite side of the Ser 90 and Lys 93, the main chain NH groups of Ser 90 and Ser 344 point out towards the surface. The side chain of a second conserved Lysine residue, Lys 342, has its ammonium group in the wall at the closed end of the depression. These lysines have most of their side chains buried in the protein molecule. However, movement about the side chain is possible for both of them (as shown by dynamics simulations).

Ser 90 is situated at the amino terminus of the buried central helix of the helical domain, suggesting a role for the dipole of this helix in the catalytic mechanism. The large cavity in the structure adjacent to this helix (cave) lies below the depression with its closest approach to the surface almost beneath Ser 90

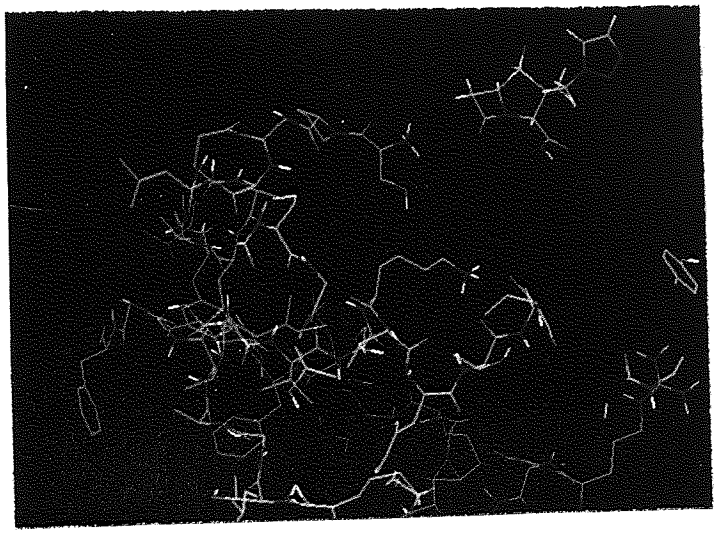
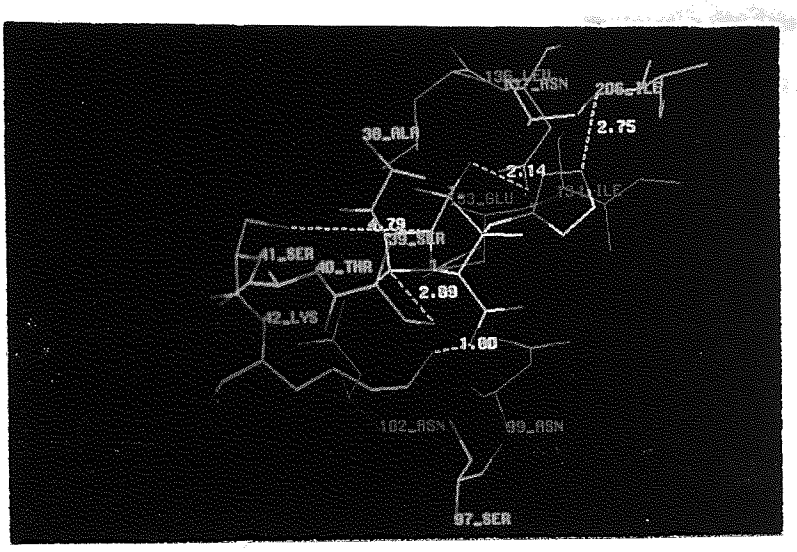


Figure 39. Predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. Predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} with a molecule of MOPAC minimised tazobactam positioned just outside the active site cleft displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Items are colour coded as follows; carbon atoms green, sulphur = yellow, oxygen = red, nitrogen = blue, hydrogen = white.

a



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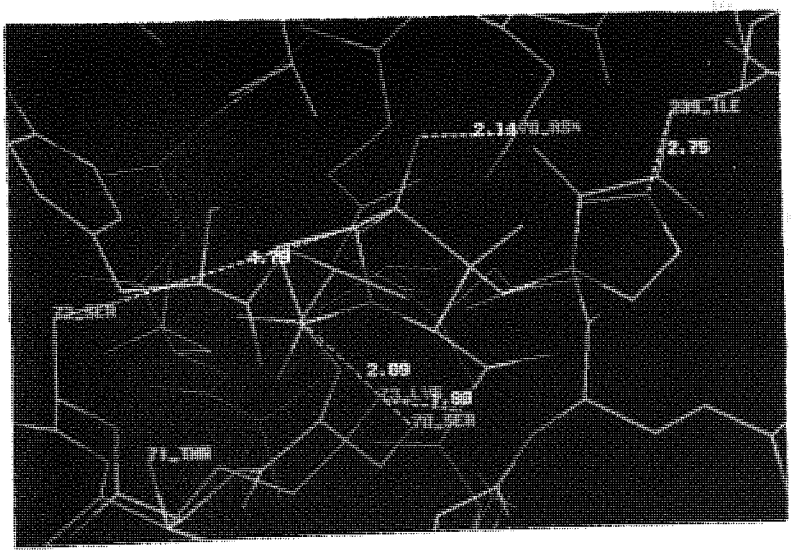


Figure 40. MOPAC minimised structure of tazobactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. Predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} with a molecule of MOPAC minimised tazobactam docked into it displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Distances between possible contact residue side chains and tazobactam are shown. Figure 40b shows possible alternative contacts. Items are colour coded as follows; carbon atoms green, sulphur = yellow, oxygen = red, nitrogen = blue, hydrogen = white.

Figure 40 shows a molecule of tazobactam docked into the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase. The distance of possible contacts between the molecule of tazobactam and residue side chains are shown. Careful examination of the figure shows that it may be the α-face of the tazobactam molecule which is presented to the reactive serine. The serine hydroxyl is positioned about 3.5Å from the carbonyl carbon of the β-lactam ring. In preparation for nucleophilic attack on the carbonyl, either enzyme can polarize the carbonyl bond by forming hydrogen bonds with two main chain amides of serine 90 and that of the β-strand amino acid at position Ser 345, 3.4Å, just after the conserved tripeptide sequence Lys-Thr-Gly. This proton lability may be assisted by a positively charged lysine, whose amino group lies within approximately 3Å of the hydroxyl group.

After attack by Ser 90, the newly formed acyl-enzyme intermediate and the opening of the β-lactam ring, the carbonyl oxygen of the newly formed acyl-group may form hydrogen-bonds to the two NH groups of Ser 90 and Ser 345 forming a sort of oxyanion hole. While this kind of hydrogen-bond-induced oxyanion hole has been seen in the serine protease group of enzymes it should be noted that the β-lactamases do not contain a catalytic histidine within a charged transfer triad Asp-His-Ser. Figure shows that tazobactam lies within bonding distance of Ser 90 NH group and Ser 345 NH group. The acyl-amino NH of tazobactam may then form a hydrogen bond with the carbonyl oxygen of Ser 345 which may then cause a conformational change in the broken β-lactam which might possibly be a rotation around the C3-C4 and N1-C4 bonds. What may in future prove to be significant is the number of possible hydrogen bonds which can be formed by the conserved lysines (Lys 93 and Lys 342), Ser 90, Tyr 177 and Asn 179.

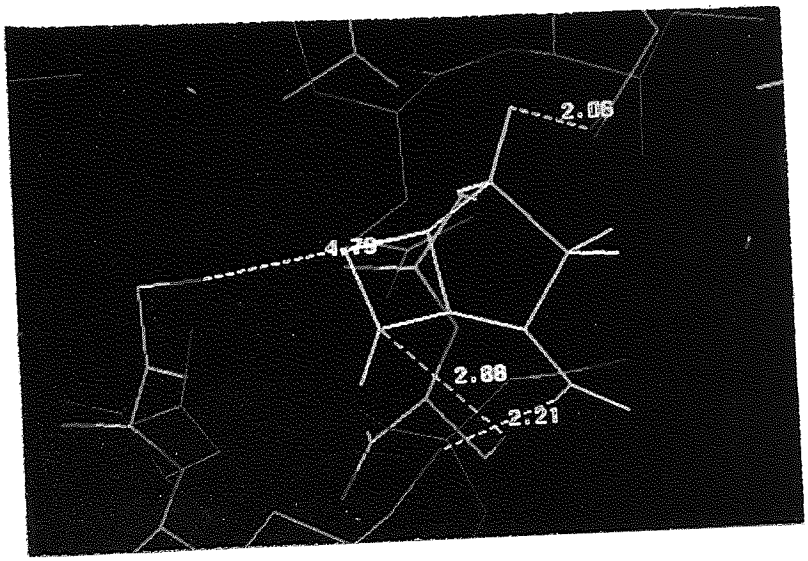
Possibly Tyr 177 may form an anion which then acts as a general base during β-lactam hydrolysis in the same way as the active site His 57 does in the serine proteases.

8.9 Interaction of sulbactam with the active site of *P. aeruginosa* SAI^{const} chromosomal β-lactamase.

Like tazobactam, sulbactam might be an irreversible inhibitor of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. This suggests that as with

tazobactam, sulbactam should be positioned so as to interact with the active site of the enzyme.

a



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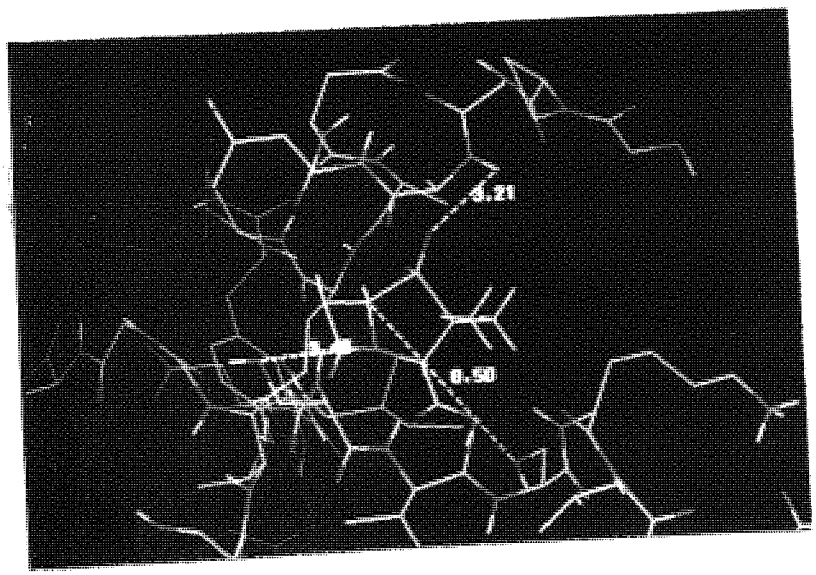


Figure 41. MOPAC minimised structure of sulbactam docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. Predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} with a molecule of MOPAC minimised sulbactam docked into it displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Distances between possible contact residue side chains and sulbactam are shown. Figure 41b shows possible alternative contacts. Items are colour coded as follows; carbon atoms green, sulphur = yellow, oxygen = red, nitrogen = blue, hydrogen = white.

Figure 41 shows a molecule of sulbactam docked into the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase. The distance of possible contacts between the molecule of sulbactam and residue side chains are shown. Probably the most important interaction between the β-lactamase and sulbactam is that which involves the sulphone oxygens. The sulphoxide of sulbactam is no good as an inhibitor so the sulphone interaction must be vital in sulbactam's role as an inhibitor (Labischinski *et al.*, 1987). It may be the interaction of the sulphone group in conjunction with the triazole group interactions (which is lacked by sulbactam) which is responsible for the enhanced activity of tazobactam over sulbactam.

The sulphone group is positioned so that its oxygens lie within 3.21Å of the NH group of the polypeptide backbone near the DAE motif. A similar interaction is seen with tazobactam. The serine hydroxyl is positioned about approximately 3.0 Å from the carbonyl carbon of the β-lactam ring. In preparation for nucleophilic attack on the carbonyl, the enzyme can polarize the carbonyl bond by forming hydrogen bonds with two main chain amides of serine 90 and that of the β-strand amino acid at position Ser 345, 3.0 Å, just after the conserved tripeptide sequence Lys-Thr-Gly. This proton lability may be assisted by a positively charged lysine, whose amino group lies within approximately 3Å of the hydroxyl group. Sulbactam lies within bonding distance of Ser 90 NH group and Ser 345 NH group. The acyl-amino NH of sulbactam may then form a hydrogen bond with the carbonyl oxygen of Ser 345. Ser 92 also lies within 3.46Å of the β-lactam carbonyl but 8.5Å away from C5, however, the dynamic simulations carried out in section 8.6 show that movement of the polypeptide chain does bring Ser 92 within an interactive distance with the C5 of the β-lactam.

This particular docking of sulbactam makes it difficult for sulbactam to form hydrogen bonds with the conserved lysines (Lys 93 and Lys 342) or perhaps with Tyr 177 and Asn 179, like those formed between these residues and tazobactam, which may be important for the stabilisation of the enzyme inhibitor complex. Sulbactam also lacks the triazole moiety, which may form stabilising bonds with the enzyme in the case of tazobactam. This might perhaps be responsible for the enhanced activity of tazobactam over sulbactam

8.10 Interaction of clavulanic acid with the active site of the β-lactamase of *P. aeruginosa* SAI^{const}.

Clavulanic acid like sulbactam and tazobactam may behave as a progressive irreversible inhibitor of the *P. aeruginosa* SAI^{const} β-lactamase. This suggested that clavulanic acid should be docked into the active site of the enzyme. The results of the docking are shown in Figure 42.

Figure 42 shows a molecule of clavulanic acid docked into the active site of the *P. aeruginosa* SAI^{const} chromosomal β-lactamase. The distance of possible contacts between the molecule of clavulanic acid and residue side chains are shown. Ser 90 is not favourably positioned so as to attack the β-lactam bond, being approximately 4.9Å away. However, the β-lactam bond is positioned approximately 3.1Å from Ser 92 and the substituent side chain approximately 3.9Å from Lys 93, which may effectively hold the molecule in the active site so blocking it. The interaction of the sulphone oxygens, which is thought to partly explain the enhanced activity of tazobactam, does not exist for clavulanic acid.

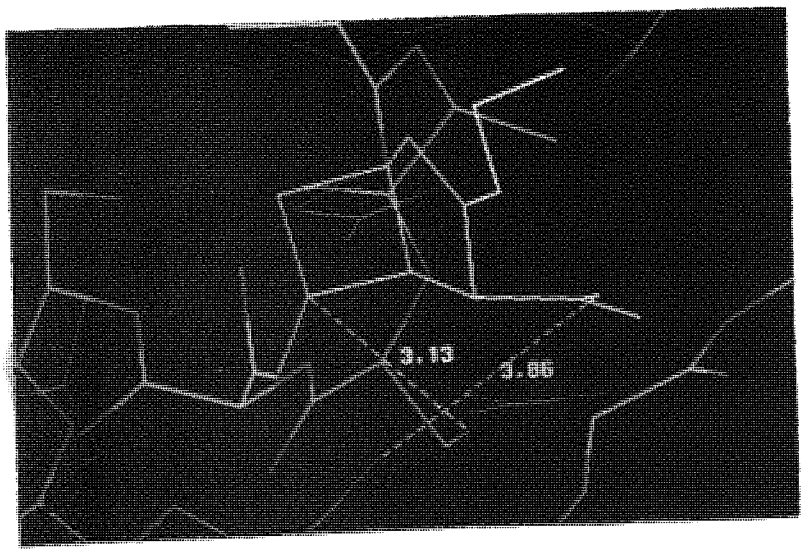


Figure 42. MOPAC minimised structure of clavulanic acid docked into the predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const}. Predicted active site of the chromosomal β-lactamase of *P. aeruginosa* SAI^{const} with a molecule of MOPAC minimised clavulanic acid docked into it displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Distances between possible contact residue side chains and clavulanic acid are shown. Items are colour coded as follows; carbon atoms green, sulphur = yellow, oxygen = red, nitrogen = blue, hydrogen = white.

8.11 Interaction of tazobactam with *S. aureus* PC1 class A β-lactamase active site.

The results obtained in section 6 suggest that tazobactam acts as a progressive but reversible inhibitor of the *S. aureus* PC1 β-lactamase but is a possible irreversible covalently binding inhibitor of the β-lactamase of *P. aeruginosa* SAI^{const}. As tazobactam showed competitive inhibition kinetics with the *S. aureus* PC1 β-lactamase (suggesting the inhibitor binds at the active site), the Knowles model was used to dock tazobactam into the active site of the enzyme. The results of the docking are shown in Figure 43.

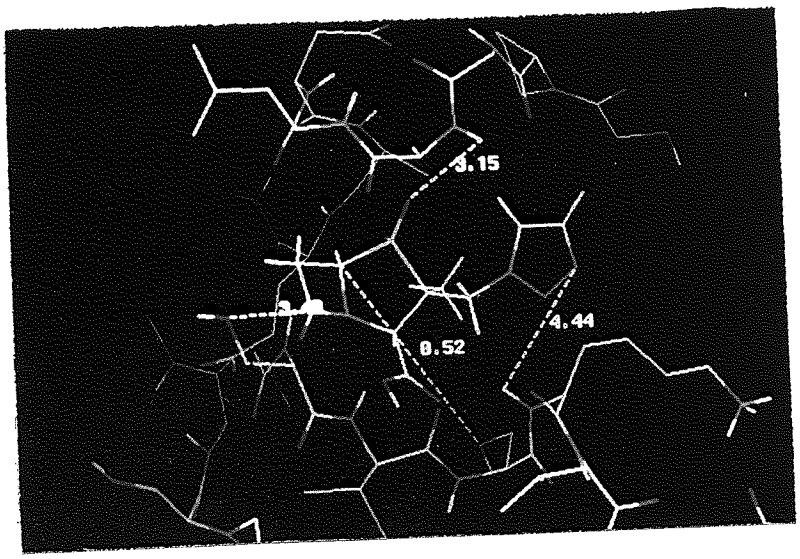
Figure 43 shows a molecule of tazobactam docked into the active site of the *S. aureus* PC1 class A β-lactamase. The distance of possible contacts between the molecule of tazobactam and residue side chains are shown. Examination of the figure shows that it may be the α-face of the tazobactam molecule which is presented to the reactive serine. The reactive serine lies in the crevice of the enzyme at a junction between an area of α-helix and β-sheet. The NH terminus of the α-helix makes a favourable region for a negatively charged molecules (like a β-lactam) because of the dipole which is associated with the helix. The serine hydroxyl is positioned about 2.8Å from the carbonyl carbon of the β-lactam ring. The active site is also bounded by several other residues Val 88 and Ile 302/3. Immediately opposite the side chain of Ser 62 is the positively charged ammonium group of Lys 65, which is also well positioned to take part in the reaction at approximately 3.5Å from tazobactam, and which may be capable of forming a salt bridge with the negatively charged carboxylate group of Glu 225.

Before Ser 62 the main chain NH groups of Ser 62 and Gln 301 point out of the surface of the active site groove. The side chain of the second conserved lysine, Lys 298, projects its ammonium group into the wall at the end of the active site. Two conserved asparagine side chains, Asn 164 and Asn 230 jut out into the active site near Glu 225 and Ile 302/3.

In order for bonding to take place the carbonyl-carbon of the β-lactam ring must come to lie close to the O of Ser 62 OH group so that the acyl-enzyme may be formed. In preparation for nucleophilic attack on the carbonyl, either enzyme can polarize the carbonyl bond by forming hydrogen bonds with the two main chain amides of Ser 62

and that of the β-strand amino acid at position Gln 301, just after the conserved tripeptide sequence KSG. The carboxyl group of the β-lactam ring must then make a favourable electrostatic interaction with the enzyme, possibly by Lys 298's ammonium side chain which lies within approximately 3Å of the hydroxyl group.

a



b

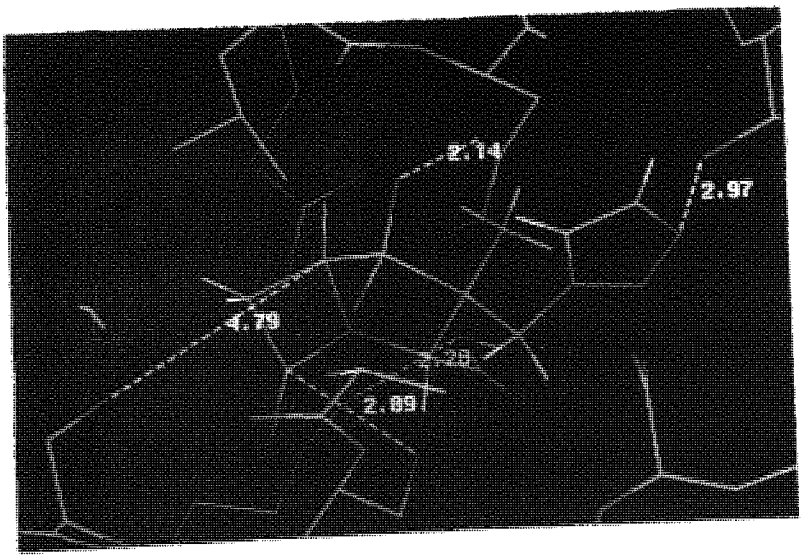


Figure 43. MOPAC minimised structure of tazobactam docked into the crystal structure active site of the class A β-lactamase of *S.aureus* PC1. Crystal structure active site of the class A β-lactamase of *S. aureus* SAI^{const} with a molecule of MOPAC minimised tazobactam docked into it displayed in QUANTA format on Silicon Graphics Iris 3120 workstation. Distances between possible contact residue side chains and tazobactam are shown. Figure 43b shows possible alternative contacts. Items are colour coded as follows; carbon atoms green, sulphur = yellow, oxygen = red, nitrogen = blue, hydrogen = white.

The β-lactam carbonyl oxygen may then come to lie over the two exposed peptide backbone NH groups of Ser 62 and Gln 301. The Lys 65 side chain itself does not make any direct electrostatic interactions with the β-lactam but acts indirectly via a salt bridge which it is capable of forming with Glu 225, giving a suitable side chain environment and allowing the interaction of Ser 62 and Gln 301 NH groups with the β-lactam carbonyl oxygen. Possibly the Val 88 and Ile 302/3, which are capable of participating in hydrophobic interactions because of their basic nature, may also interact with substituent groups on the basic β-lactam molecule such as the triazole group of tazobactam. Ile 302/3 is positioned approximately 2.75Å from the triazole ring of tazobactam whilst Val 88 is 3.2Å away (just out of picture). The link of the β-lactam substituent group may form hydrogen bonds between the carbonyl oxygen and the side chain of Asn 164 which is approximately 2.2Å away from this group and its amide group and the peptide carbonyl of Gln 301 which is 2.86Å away (just out of picture), thus creating a favourable electrostatic environment for further reactions. The substituent group of the β-lactam might also be capable of forming some kind of electrostatic interaction with the side chain of Asn 230 (2.14Å from β-lactam carbonyl oxygen) and the peptide carbonyl oxygen of Asn 164 (2.34Å from β-lactam carbonyl oxygen). An important factor which might govern the interactions formed could be the type of substituent group on the β-lactam. If the substituent group is very large or particularly inflexible then this might affect the ability of the β-lactamase to hydrolyse it. For instance, if the secondary reaction depends on a conformational change in the β-lactam taking place after the initial nucleophilic attack, then if the side chain, in some way interacts with the enzyme so preventing this necessary change perhaps by holding the β-lactam molecule in some unfavourable conformation then the hydrolysis of the β-lactam may be significantly slowed or even prevented.

8.12 Proposed mechanism of substrate docking and interaction with β-lactamase

A conclusion from the many studies of enzyme-substrate interactions is the importance of the role played by electrostatic forces in determining the orientation of molecules in the complex (Weinstein *et al.*, 1985). This is not surprising in view of the long range of the electrostatic forces compared to other intermolecular forces (Rigby *et*

al., 1986), and the special significance of electrostatic interactions in the structure and function of proteins (Warshel and Russell, 1984; Honig *et al.*, 1986) and especially in enzymes (Russell *et al.*, 1987; Wells *et al.*, 1987).

The electrostatic surface potential is an important control on the docking of any molecule to another. Before a substrate molecule can dock into the active site of an enzyme it must be electrostatically and sterically complementary to the docking site. This specific type of complementarity occurs at only one particular place on the enzyme which means that it would be impossible to dock a substrate molecule at any position other than the active site because the lack of electrostatic and steric complementarity would result in an unfavourably high binding energy and the correspondingly high energy of interaction would cause the repulsion of the substrate molecule from the enzymes surface. The docking position used in the modelling studies for tazobactam, sulbactam and clavulanic acid was favoured because it gave the lowest energy of interaction of any chosen position in the protein structure.

A substrate is guided towards the active site groove due to interactions between chemical groups on the substrate and the amino acids on the surface. The surface of the protein is composed of regions of hydrophobic and hydrophilic electrostatic potential. The residue groups contributing to these regions do not necessarily occur close to one another in the amino acid sequence of the enzyme but are oriented close to each other upon protein folding. Individual amino acids may also contribute to both hydrophobic and hydrophilic areas. In solution the substrate is gradually guided into the active site groove and finally orientated into the correct position through interactions with amino acid side chains. Interaction with other areas of the protein is prohibited by high energy of interaction. A high energy of interaction repels the substrate molecule from the surface of the protein. As the substrate approaches the active site groove the energy of interaction becomes more favourable, the interaction energy drops and the substrate is no longer repelled by the surface. The energy of interaction is lowest when the substrate is bound in the active site. As the substrate approaches the active site the interaction with certain residues correctly orientates the substrate with regard to the key catalytic residues.

The important segments of polypeptide that come together to form the penicillin-

binding site of the class C *P. aeruginosa* SAI^{const} and class A *S. aureus* β-lactamases were modelled in section 8.5 and show that a number of the conserved residues are close to the antibiotic binding site; these being Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*), Lys 93 (*P. aeruginosa*) or Lys 65, (*S. aureus*), Tyr 177(*P. aeruginosa*) or Ser 160 (*S. aureus*), Asp 245 (*P. aeruginosa*) or Glu 225 (*S. aureus*), Leu 229 (*S. aureus* only), Lys 342 (*P. aeruginosa*) or Lys 298 (*S. aureus*), and Gly 344 (*P. aeruginosa*) or Gly 300 (*S. aureus*). Many of the conserved residues are contained in or bordering the long strand from 86 to 111 (*P. aeruginosa*) and 58-83 (*S. aureus*), which leads into and through the second α-helix (α2 helix). Around the conserved Leu 73 (*S. aureus* PC1 β-lactamase) is a triad of three conserved leucines,78/79, 254 and 260, (Lys 108, Leu 266 and Leu 279 in *P. aeruginosa* β-lactamase) which may help to anchor the C-terminal end of the important a2-helix. The two invariant prolines (92 and 312,*S. aureus* and 120 and 357, *P. aeruginosa*) are near turn positions of the chain.

The β-lactam substrate probably approaches the active site which is at the N-terminus of the α2 helix from the bottom or lower front of the enzyme as the calculated interaction energies between a series of enzyme residues and the β-lactam are lower in this region of the groove then those for a series of enzyme residues and the β-lactam from the upper region of the groove. Prior to and within this helix is the conserved sequence Phe 86xxxxSerxxLys 93 (*P. aeruginosa*), Phe 58xxxxSerxxLys 65 (*S. aureus*), which is present in all class C and class A enzymes as well as some PBP sequences (Frere and Joris, 1985). Phe 86 (*P. aeruginosa*) or Phe 58 (*S. aureus*) is a long way from the reactive Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) (approximately 14Å) and lies against the back face of the β-sheet. The conserved Lys 73 (*P. aeruginosa*) or Lys 65 (*S. aureus*) in this sequence is actually adjacent to Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) because of the geometric constraints of an α-helix. This places the two residues within hydrogen bonding distance.

Forming the right side of the binding site is the b3 strand, which contains the recurring sequence 341-344 (*P. aeruginosa*) or 297-300 (*S. aureus*) (Asp in *S. aureus* PC1 and Asn in *P. aeruginosa*) Lys 342 (*P. aeruginosa*) or Lys 298 (*S. aureus*), Thr (Ser 299 in *S. aureus*) 343, Gly 344 (*P. aeruginosa*) or Gly 300 (*S. aureus*). This structural motif exists in several equivalent forms in all penicillin-recognising enzymes (Kelly *et*

al., 1987). The ε N of Lys 342 (*P. aeruginosa*) or Lys 298 (*S. aureus*) is at the upper right of the catalytic site far (approximately 4.8Å) from Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) hydroxyl, and may be hydrogen bonded to the conserved Ser 160 (*S. aureus*) or Tyr 177 (*P. aeruginosa*). The next residue in the β-strand at position 343 (*P. aeruginosa*) or 299 (*S. aureus*) is, in all sequences a potential hydrogen binder, usually a Ser (as in *S. aureus* PC1) or a Thr (as in *P. aeruginosa*). The conserved glycine at 344 (*P. aeruginosa*) or 300 (*S. aureus*) is the residue closest to Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) and is found to occupy this position in all β-lactamases as well as PBPs (this residue may be important in substrate specificity e.g. TEM plasmid β-lactamase (Hall and Knowles, 1976)).

In the class A *S. aureus* β-lactamase at the bottom of the binding site is the α7 helix and loop which contains three important residues. These are the invariant Arg 223, Glu 225, and Leu 229. Arg 223 projects into the solvent, the conserved Glu 225 is associated with the active site by a weak interaction with Lys 65 at approximately 3Å, and a stronger one with Asn 230 at 2.9Å, which does not have an apparent equivalent in the class C β-lactamases. In the class C *P. aeruginosa* β-lactamase there is an insertion between residues 219-220 of the *S. aureus* sequence and a deletion between residues 228-231 of the *S. aureus* sequence in the residues at the bottom of the binding site. This is the area where β-lactam side chains would be expected to lie. The equivalent of the class A *S. aureus* Glu 225 (Asp 245, *P. aeruginosa*) is now positioned so as to be more distant from the acyl intermediate and although the overall positions of the peptide backbone which delimits the active site does not change, the effect of the change in residue side chains means that more room is available for β-lactam substrates.

Two short segments of polypeptide chain exist at the left of the binding site. One segment is the inter-helix loop 172-178 (*P. aeruginosa*) or 155-163 (*S. aureus*). The loop contains the conserved Tyr 177(*P. aeruginosa*) or Ser 160 (*S. aureus*) and Ser 178 (*P. aeruginosa*) or Asp 163 (*S. aureus*), the former being near three important residues Ser 90, Lys 93 and Lys 342 (*P. aeruginosa*) or Ser 62, Lys 65 and Lys 298 (*S. aureus*). The hydrogen bonding of Tyr 177 (*P. aeruginosa*) or Ser 160 (*S. aureus*) and Lys 342(*P. aeruginosa*) or Lys 298 (*S. aureus*) may help the correct positioning of the substrate binding strand β3 strand relative to the catalytic α2 helix. The Ser 178 (*P. aeruginosa*) or Asp 131 (*S. aureus*) is directed away from the catalytic residues. Asn

179 (*P. aeruginosa*) or Asn 164 (*S. aureus*) projects into the binding site and may interact with β-lactam substrates. The second segment, the loop 114 to 126 (*P. aeruginosa*) or 84 to 100 (*S. aureus*) on the lower front face of the molecule places another functionality, Arg 114 (*P. aeruginosa*) or Asn 84 (*S. aureus*) near the bound substrate.

The substrate is most likely orientated so that the thiazolidine ring is at the top and the acylamido substituent at the bottom of the site. The β-lactam could initially be attracted by the exposed Gly 350 (*P. aeruginosa*) or Arg 306 (*S. aureus*) on the b3 strand. After the preliminary attraction, the carboxyl group could experience additional attractions from Lys 342 (*P. aeruginosa*) or Lys 298 (*S. aureus*), which is more exposed than Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*) and also hydrogen binding from the nearby Thr 343 (*P. aeruginosa*) or Ser 299 (*S. aureus*). The carbonyl bond of the β-lactam ring and the amide bond of the acylamido linkage are positioned to hydrogen bond in an anti-parallel fashion to the backbone amide and carbonyl groups of residue Thr 343 (*P. aeruginosa*) or Ser 299 (*S. aureus*), the side chain of which need not be and is usually not conserved between different groups of β-lactamases. In this orientation the amide of Thr 343 (*P. aeruginosa*) or Ser 299 (*S. aureus*) and the partial positive charge of the α2 helix dipole form part of the oxyanion hole (Kraut, 1977). The amide of Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*)can also attract the β-lactam carbonyl, and the amide therefore completes the formation of the oxyanion hole as suggested by Herzberg and Moult (1987). With the β-lactam carbonyl and ring carbonyl thus fixed, the β-lactam ring is aligned for an α-face attack (Boyd, 1982) by Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*). An alternative ion-pair interaction of the β-lactam's carboxylate solely with the Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*) in the conserved SerxxLys peptide has been postulated in PBPs (Boyd, 1987; Varetto *et al.*, 1987).

An acylamido substituent would lie a the bottom of the binding site. Asn 230 (*S. aureus*) sticks upward from the α7 helix, the substituent must remain fairly exposed alongside the β3 strand. (A similar exposed position for acylamide substituents was seen experimentally for β-lactam complexes with the *Streptomyces* R61 DD carboxypeptidase (Kelly *et al.*, 1989)). A result is the ability of β-lactamases in general to hydrolyse β-lactams with a variety of very large substituents beyond the acylamide. Perhaps this is why the *S. aureus* PC1 β-lactamase with a unique insertion at 346-347

in the *P. aeruginosa* sequence on the b3 strand of an isoleucine side chain is less effective in hydrolysing β-lactams with branched side chains at the α-carbon of the acylamide (Coulson, 1985; Pollock, 1968; Citri and Pollock, 1966; Naylor, 1971).

In the mechanism of interaction of sulbactam with the active site of the TEM (class A) β-lactamase as proposed by Knowles (1985), the active site serine is positioned to attack the β-lactam carbonyl carbon atom of sulbactam whilst the carboxylic acid substituent is presumed to be associated with the lysine (93, *P. aeruginosa*; 65, *S. aureus*) residue of the SxxK motif, which is common to all β-lactam interacting proteins. After cleavage of the β-lactam bond and rearrangement of the fragment, a second nucleophilic attack is proposed leading to a second covalent linkage at the C5 position of the inhibitor. The enzyme residue (X) involved in this second attack has not been identified. The Knowles model was used to dock tazobactam into the active site of the model structure. The SxxK motif is positioned to interact as proposed in the Knowles model allowing the Serine 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) hydroxyl to attack the β-lactam carbonyl. The serine hydroxyl group could be activated in at least two ways. First, the dipole moment of the α-helix provides a formal half positive charge (Hol, 1985) which might help to lower the pK of the hydroxyl proton. A similar effect is seen in papain where the reactive Cys 25 is at the N-terminus of a helix (Drenth *et al.*, 1976). Second, the charged amino group of Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*), only 2.8Å from Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*), may repel and orient the serine's proton for direct transfer to the β-lactam during formation of the acyl intermediate. A proton relay path via another residue is not obvious from this model. β-Lactamase is unlike the classical serine enzymes in that no histidine is available for based-catalysed activation of the serine hydroxyl which is what happens in the serine proteases. The carboxyl of the glutamic acid residue is about 3.6 and 3.3Å from Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) and Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*), respectively. Glu 225 (*S. aureus* β-lactamase) or Asp 245 (*P. aeruginosa* β-lactamase) could play a direct role in base catalysis if it is brought closer to Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*)by conformational changes or vibrational modes. β-Lactamases have been reported to be quite flexible (Fink, 1985), this together with the results from the dynamics on the individual sections of the active site suggest that Glu 225 (*S. aureus* PC1 β-lactamase) or Asp 245 (*P. aeruginosa* β-lactamase) could

be brought sufficiently close to Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) for it to play a part in base catalysis. Glu 225 (*S. aureus* β-lactamase) or Asp 245 (*P. aeruginosa* β-lactamase) could also assist in deacylation by hydrogen bonding to an approaching water molecule prior to nucleophilic attack as has been suggested for the *S. aureus* PC1 β-lactamase (Herzberg and Moult, 1987) though many other groups are available for this role (Asn 84, Asn 164 and Asn 230) (Arg 114, Ile 182 and Gly 248 in *P. aeruginosa* β-lactamase).

The polarisation of the β-lactam carbonyl bond prior to nucleophilic attack by Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) is most likely accomplished by hydrogen bonding to the backbone amides of Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*)and Thr 346 (Ala 302 in *S. aureus* PC1 β-lactamase) or Asp 245 (*P. aeruginosa* SAI^{const} β-lactamase), leading to α-face attack on the β-lactam. To a lesser extent, the conserved Tyr 177 (*P. aeruginosa*) or Ser 130 (*S. aureus*) could conceivably polarise the β-lactam carbonyl bond, but in this particular model it is difficult to position the β-lactam so that both Ser 90 (*P. aeruginosa*) or Ser 62 (*S. aureus*) and Tyr 177 (*P. aeruginosa*) or Ser 299 (*S. aureus*) Ser 160 are correctly orientated with respect to the β-lactam carbonyl bond.

If only the attractions utilising the C3 carboxyl and β-lactam carbonyl are sufficient for initial recognition, that is, that the acylamido amide hydrogen bond to the backbone carbonyl of residue Thr 346 (*P. aeruginosa* β-lactamase) or Ala 302 (*S. aureus* PC1 β-lactamase) is not required, then the binding of β-lactam without C6 substituents, such as penicillanic acid sulphone or clavulanic acid, can be better understood. The variable side chain of residue 346(*P. aeruginosa*) or 302 (*S. aureus*) could contact large β-face substituents at C2 or the β-oxygen of sulphones. β-Halo substituents at C6 are more distant from 346 (*P. aeruginosa*) or 302 (*S. aureus*), but may be in contact with larger enzymatic side chains at position 346 (*P. aeruginosa*) or 302 (*S. aureus*). This system does not actually use the nitrogen of the β-lactam ring but this may help to explain the binding of cyclobutanones to the carboxypeptidases and β-lactamases (Kelly *et al.*, 1985; Lowe and Swain, 1985).

Jones *et al.* (1989) suggested that the ring nitrogen of a penicilloate inhibitor is directly protonated by Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*)but this is

inconsistent with the distance between the nitrogen and the lysine (5.8Å). Arg 359 (*P. aeruginosa*) or Arg 306 (*S. aureus*) could be a possible a candidate in β-lactamase inactivation. It is located near the ring's carboxylate group, it is the most likely candidate as the base in the reaction of β-lactamases with sulphone or clavulanate-type inhibitors (Knowles 1985; Pratt, 1988). Clavulanates's reactive double bond at C2 would be about 3Å from Arg 244, which is much closer than either Lys 93 (*P. aeruginosa*) or Lys 65 (*S. aureus*) or Lys 342 (*P. aeruginosa*) or Lys 298 (*S. aureus*). The only known β-lactamase without arginine at this position (*S. albus* G with Asn) is less effectively inhibited by clavulanate (Frere *et al.*, 1982).

Although the active site geometry of the β-lactamases is identical to the carboxypeptidase, the active site of the carboxypeptidase active site is more hydrophobic (Knox and Kelly, 1989) which means that the hydrolysis of acyl-intermediates could be hindered. This might help to explain the slower overall reaction rate of these enzymes when compared to the β-lactamases.

A number of site-directed mutagenesis experiments help support the idea that the it is the difference in amino acid sequence both around the active site and in other areas of the protein which are responsible for the differences in substrate specificity. The β–lactamase of *B. licheniformis* strain 749/C, and another β–lactamase from strain 6346/C have been well characterised (Pollock, 1967;1968; Naylor, 1971) and the substrate profiles of the two enzymes found to be very different (Pollock, 1968; Naylor, 1971). The only difference between the two enzymes is a five residue substitution; Gln for Arg 191, Val for Met 287, Glu for Asn 293, Ser for Gly 294 and another change thought to be in the N-terminal region (Thatcher, 1975). Substitution of the conserved Lys 234 with glutamic acid resulted in a mutant with an increased K_m and a 200-fold decrease in V_{max} which also helps support the idea that a positive group at this position is involved in the attraction or orientation of the C3 carboxyl group of β-lactams, or the sulphate group of monobactams. Madgewick and Waley (1987) changed the conserved Lys 73 for an arginine which resulted in a significant loss of activity in β-lactamase I from *B. cereus* 569/H. The charge on an arginine residue tends to be more diffuse than it is on a lysine residue which could mean that it is not as well able to orientate or polarise the proton of Ser 70. The change of Glu 166 for a Gln also leads to loss of activity which may suggest that the Glu 166 is involved in the deprotonation of Ser 70 in deacylation.

Increased resistance to cephalosporin C in a TEM β-lactamase variant has been postulated as being due to the substitution of an alanine at position 237. This residue occupies a critical position in the active site of the β-lactamase because its main chain atoms can hydrogen bond to the β-lactam, and the side chain of residue 237 would be close to the thiazolidine ring of the β-lactam.

A conversion of the active site Ser-Thr to Thr-Ser in the class A β-lactamases (Dalbadie-McFarland *et al.*, 1982) causes a loss in activity probably because the serine must be displaced by at least 3.6Å to the topside of the helix, which then makes it too far away from a substrate anchored to the β3 strand. Schultz and Richards (1986) replaced Thr 71 with all possible amino acids and found that only substitutions with Tyr, Trp, Phe, Arg, Lys or Asp caused loss of activity with ampicillin, benzylpenicillin or 6-aminopenicillanic acid. This suggests that none of the substitutions at Thr 71 would sterically prevent substrate entry and binding, but because Thr 71 is buried in the enzyme between the α2 helix and the β3 strand, imposition of larger or charged side chains into the confined volume could cause a movement of the helix α2 relative to the substrate binding β3 strand. Alanine 69 is a much better candidate for substitution because it is on the substrate side of the serine containing helix, rather than behind the helix as is Thr 71. Oliphant and Struhl (1990) have reported several TEM mutants at position 69 which have an increased resistance to clavulanic acid and sulbactam.

The two features of the tazobactam structure which could be important in the interaction with the active site residues are the sulphone oxygens and the nitrogens in the triazole ring. The sulphone oxygens make favourable contacts with the NH hydrogen atoms of the peptide backbone at residues Proline 163 and Leucine 164 in (*P. aeruginosa* β-lactamase) allowing stabilisation of binding of the inhibitor through hydrogen bonding. Studies on the crystal structure of human leukocyte elastase and a cephalosporin sulphone ester have identified important hydrogen bonding to the sulphone oxygens (Navia *et al.*, 1987). The lack of activity of clavulanic acid against the class C enzymes could be due to its inability to participate in such stabilising H-bonding. The triazole ring of tazobactam is also positioned to make favourable hydrogen bonding contacts. The strong electron density surrounding the ring nitrogens (especially N3) could act as a hydrogen bond acceptor with the NH hydrogen atom of the peptide backbone between Serine 72 and Lysine 73. Hydrogen bonding could

involve the N3 atom of the triazole or a general π interaction with the triazole ring system. In support of the former mechanism a hydrogen bond in the tazobactam crystal structure between the carboxylic acid of one independent molecule and the N3 on the triazole of the other independent molecule was identified. These enzyme-triazole interactions could be responsible for the enhanced activity of tazobactam compared to sulbactam. Inspection of the active site model shows that Serine 72 is positioned 9.8Å from the C5 of tazobactam. No other residue capable of nucleophilic attack on C5 is more favourably positioned to attack C5 and it is therefore proposed that residue X is Serine 72. Formal proof still requires isolation and amino acid sequencing of a labelled tryptic fragment.

8.13 Conclusions from the modelling studies

The studies described in this chapter help in the identification of residues involved in the interaction between tazobactam and the *P. aeruginosa* SAI^{const} β-lactamase. The affinity labelling studies indicated that a tyrosine residue could be involved in the active site. The molecular graphics approach identified a tyrosine residue (in the TYAPGSQRLYS box) which was remote in the amino acid sequence from the SVSK active site region. This tyrosine residue might play an important role in the acylation of the active site serine by tazobactam. Such observations were only possible by application of biochemical and molecular graphics techniques. Similarly a lysine residue in the RLLNKTGST box together with a lysine in the SVSK box could help in substrate positioning in the active site so that the β-lactam bond is favourably positioned to interact with serine 90. This in turn might allow the serine 92 to make a second nucleophilic attack on the β-lactam ring, as proposed by Knowles.

The affinity labelling studies also identified a histidine residue in the vicinity of the active site. The exact role of this residue awaits further characterisation.

Another feature of the molecular graphics study concerns the possible interaction of lysine 73 with the carboxylic acid of tazobactam. Although the static docking shows the amino group of this lysine residue to be too far away to participate in a favourable interaction with the carboxylic acid, the dynamics simulations show it is highly flexible, and can easily move close to this residue. Although further conclusions may be

tenuous, it is tempting to conclude that this flexible lysine group participates in steering the substrate into the active site and possibly removing it after hydrolysis.

CHAPTER 9

9 FINAL CONCLUSIONS

Antibiotic inactivating enzymes are probably the most clinically relevant antibiotic resistance mechanism developed by bacteria. Treatment failures of *P. aeruginosa* lung infections in chronic CF sufferers are linked with strains in which the control of β-lactamase production has been stably partially or fully derepressed. These strains naturally overproduce the enzyme, resulting in a reduction of the efficacy of β-lactam antibiotic therapy. The proliferation of *P. aeruginosa* in the lung is favoured by conditions which enhance the production of β-lactamase and the organisms resistance to antimicrobials.

Iron is known to play a vital role in infection. It has been found to be an important environmental signal which coordinately regulates the expression of a number of virulence and metabolic genes. The results of this study suggest that the response of β-lactamase hyper-producing mutants to the conditions of iron depletion found in the CF lung may be a major contributing factor to the pathogenicity of the organisms. Cells grown in iron-depleted conditions produce elevated levels of β-lactamase compared to those grown in iron-replete conditions. Hence the iron-deplete CF lung stimulates the increased production of β-lactamase. The complicated relationship between iron-depletion, slow growth and elevated β-lactamase production enhances the resistance of the organism to β-lactam therapy.

Antibiotic resistance mediated by inactivating enzymes is a consequence of the balance between the rate of antibiotic entry into the cell and the rate of modification of the antibiotic by the enzyme or the antibiotic target by the antibiotic (Nayler, 1987). The first is related to the diffusion properties of the antibiotic and permeability barriers of the cell, the second is a consequence of the quantity of the enzyme produced by the cell and its kinetic characteristics. The major line of defence against β-lactam antibiotics is the periplasmic β-lactamase. Cells may also have two other minor lines of protection. The first is β-lactamase in the OM of hyper-producing cells. This enzyme could provide a line of defence against invading β-lactam molecules which are hydrolysed before reaching their cellular targets or may at least cause a significant decrease in the number of antibiotic molecules reaching the periplasm. The presence of this enzyme might

explain inconsistencies obtained with the Zimmermann-Rosselet permeability assays conducted with *P. aeruginosa*. The second line of defence is the secretion of β-lactamase into the medium. Secreted β-lactamase in combination with an exopolysaccharide layer or the mucoid phenotype (exhibited by cells isolated from CF lungs) which would help to localise the excreted β-lactamase in a protective zone around the cell, shield the cell from external antimicrobial agents. The overall result is the persistence of cells resistant to β-lactam therapy which are capable of establishing and continuing an infection.

Tazobactam is a very promising β-lactamase inhibitor for use in combination with β-lactamase sensitive antibacterial β-lactams for the treatment of *P. aeruginosa* infections. The results presented here suggest that tazobactam is an irreversible inhibitor of the chromosomal enzyme from *P. aeruginosa* SAI^{const}. Tazobactam also appears to be a good inhibitor of *E. cloacae* P99 β-lactamase, although kinetic studies show that its interaction with the active site of *E. cloacae* P99 β-lactamase is different from its interaction with the active site of *P. aeruginosa* SAI^{const} β-lactamase. Tazobactam also inhibits the β-lactamase of *S. aureus* PC1, although this inhibition appears to be reversible; the enzyme apparently regains its activity after an initial period of inactivation. However, the transient inhibition of this enzyme may be sufficient in clinical usage to allow the effects of a combination antibiotic to be bactericidal.

The results from the computer-modelling studies and analysis of the trypsin digested fragments of tazobactam inhibited *P. aeruginosa* SAI^{const} chromosomal β-lactamase implicate a number of residues (D T S E P G A C V I Y F H K R) in the interaction between inhibitor and enzyme. A tentative identification of the residues involved and a possible mechanism for inhibition of *P. aeruginosa* SAI^{const} β-lactamase by tazobactam involves the initial attraction of the β-lactam by the surface exposed Arg 360 residue. Lys 342 then attracts the carboxyl group and allows hydrogen bonding by Thr 343, followed by the correct orientation of the carbonyl bond of the β-lactam ring and the amide bond of the acylamido linkage for hydrogen bonding to Thr 346. The net result is the favourable positioning of the β-lactam for nucleophilic attack by Ser 90 on the β-lactam carbonyl and formation of an enzyme-inhibitor intermediate. A

possible interaction exists between the sulphone oxygens and the Asp 245, Ala 246, Glu 247 triad. There are several candidate residues for interaction with the triazole group of tazobactam (His 118 or Asn 347). Bonding between one or both of these residues to the triazole group and the sulphone oxygens to the DAE triad stabilises the acyl-enzyme intermediate and prevents rearrangement and the favourable positioning of the C5 of the β-lactam resulting in the inhibition of the β-lactamase. In a substrate β-lactam, attack on the β-lactam carbonyl is followed by a second nucleophilic attack on the β-lactam C5 either by Lys 93 (which is most favourably positioned in the active site model) or by Ser 92.

Future work could investigate the amino acid sequences of affinity labelled tryptic fragments in order to identify the precise sequence of residues involved in binding tazobactam to the active site of *P. aeruginosa* chromosomal β-lactamase. Additional affinity labelling studies could be conducted with a view to identifying the active site sequences of the β-lactamases of *S. aureus* PC1 and *E. cloacae* P99. The effects of growth rates and β-lactam levels on the production of other virulence factors could also be investigated.

In summary, the data presented in this thesis indicates that expression of β-lactamase can be modulated by growth rate, availability of iron and by the presence of β-lactams in the growth medium. The β-lactamase inhibitor tazobactam binds to the active site of the β-lactamase via a number of active site residues. Tentatively, these residues were identified as Ser 90, Ser 92, Lys 93, Asp 245, Ala 246, Glu 247, His 118 or Asn 341. A number of other residues were implicated in recognition, substrate orientation and activation of the major catalytic residues.

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