Probing the Non-Proline *Cis* Peptide Bond in β-Lactamase from *Staphylococcus aureus* PC1 by the Replacement Asn136 → Ala[†,‡]

Soojay Banerjee,[§] Nobuharu Shigematsu,[∥] Lewis K. Pannell,[∥] Sergei Ruvinov,[§] John Orban,[§] Frederick Schwarz,[⊥] and Osnat Herzberg*[,§]

Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, 9600 Gudelsky Drive, Rockville, Maryland 20850, Center for Advanced Research in Biotechnology, National Institute of Standards and Technology, 9600 Gudelsky Drive, Rockville, Maryland 20850, and Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: A non-proline *cis* peptide is present between Glu166 and Ile167 in the active site of β-lactamase from *Staphylococcus aureus* PC1. To examine the role of the interaction between the side chain of Asn136 and the main chain of Glu166, the site-directed mutant N136A was produced. The enzyme shows no measurable hydrolytic activity toward a variety of penicillins or cephalosporins except for the chromogenic cephalosporin, nitrocefin. For nitrocefin, the progress curve exhibits a fast burst with a stoichiometry of 1 mol of degraded substrate per mole of enzyme followed by a slow phase with a hydrolysis rate that is reduced by approximately 700-fold compared with that of the wild-type enzyme. Thus, the mutant enzyme is deacylation defective. Monitoring the hydrolysis of nitrocefin after preincubation with a number of β-lactam compounds shows that cephalosporins form stable acyl complexes with the enzyme, whereas penicillins do not. The molecular weight of the mutant was determined by electrospray mass spectrometry, and the presence of the stable acyl enzyme adducts with cephaloridine and cefotaxime was confirmed by both electrospray and MALDI mass spectrometry. Therefore, in addition to impairing deacylation, the acylation machinery has been altered compared with the wild-type enzyme to act on cephalosporins and not on penicillins. Urea denaturation and thermal unfolding studies show that the N136A mutant enzyme is less stable than the wild-type enzyme. However, stability against chemical denaturation of the mutant enzyme is enhanced in the presence of cephaloridine beyond the stability of the wild-type protein. This is attributed to accumulation of favorable interactions between the cephaloridine and the protein, which play a role in the folded state and not in the unfolded state.

β-Lactamases (EC 3.5.2.6) are a family of bacterial enzymes that inactivate β-lactam antibiotics by hydrolyzing the β-lactam amide bond typical of this group of compounds (Abraham & Chain, 1940). The proliferation of these enzymes among pathogenic bacteria has considerably reduced the usefulness of β-lactam antibiotic therapy. They have been extensively studied because of both their clinical importance and interesting biochemical properties (Hamilton-Miller & Smith, 1979; Coulson, 1985; Frère & Joris, 1985; Knowles, 1985; Herzberg & Moult, 1991a). There are three sequence-based classes of β-lactamases that utilize an active site serine. Of these, the class A enzymes are plasmid-encoded monomeric molecules, approximately 250 amino acid residues in length (Ambler, 1980), common in Gram-positive and Gram-negative bacteria. For the serine β-lactamases, hydrolysis of the β-lactam amide bond proceeds via a mechanism that involves an acyl enzyme intermediate at the O^{γ} atom of an invariant serine residue that acts as the nucleophile (Knott-Hunziker *et al.*, 1979; Cartwright & Coulson, 1980; Cohen & Pratt, 1980; Fisher *et al.*, 1981). Following the consensus numbering scheme of Ambler *et al.* (1991), this serine is at position 70.

A number of high-resolution crystal structures have been determined revealing that the structures of enzymes from different organisms are similar, consistent with the sequence homology (Herzberg & Moult, 1987; Herzberg, 1991; Moews *et al.*, 1990; Knox & Moews, 1991; Jelsch *et al.*, 1992, 1993; Strynadka *et al.*, 1992). In addition to Ser70, Lys73 and Glu166 have been shown to be key catalytic residues. The role of these, as well as that of Ser130 and the mechanistic similarity to the class C serine β-lactamases, has been the subject of much discussion (Herzberg & Moult, 1987, 1991a; Gibson *et al.*, 1990; Ofner *et al.*, 1990; Strynadka *et al.*, 1992; Lobkovsky *et al.*, 1994; Damblon *et al.*, 1996). An analogy to the mechanism of the serine proteases can be drawn because of the involvement of the active site serine and the possible role of an oxyanion hole in catalysis (Herzberg & Moult, 1987). Controversies arise at several levels relating to how exactly Lys73, Glu166, and Ser130 may assist catalysis, and to whether the machinery for acylation is the same as that used during deacylation (note that, throughout the paper, the term acylation refers to the chemical step of formation of the acyl enzyme from substrate

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[‡] Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does this identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material, instrument, or equipment identified is necessarily the best available for the purpose.

* To whom correspondence should be addressed. Telephone: 301-738-6245. Fax: 301-738-6255. E-mail: osnat@elan1.carb.nist.gov.

[§] University of Maryland Biotechnology Institute.

[∥] National Institutes of Health.

[⊥] National Institute of Standards and Technology.

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and free enzyme and deacylation refers to the conversion of the acyl enzyme into a free enzyme and a hydrolyzed substrate).

The class A enzymes contain a water molecule with enhanced nucleophilicity due to its interaction with Glu166. It has been proposed to be the hydrolytic water molecule (Herzberg & Moult, 1987; Herzberg, 1991). Mutagenesis and crystallographic studies support this proposal and suggest that the role of Glu166 is predominantly during deacylation. In the P54 mutant β-lactamase from *Staphylococcus aureus* PC1 (with the D179N replacement), Glu166 is conformationally disordered, and deacylation becomes the rate-limiting step (Herzberg *et al.*, 1991). In the *Escherichia coli* TEM-1 and *Bacillus licheniformis* β-lactamases, replacement of Glu166 by a noncharged residue resulted in accumulation of the acyl enzyme intermediate (Adachi *et al.*, 1991; Escobar *et al.*, 1991). One of these mutant enzymes (E166N) was used to determine the crystal structure of an acyl enzyme of the TEM-1 β-lactamase (Strynadka *et al.*, 1992). Recently, the mutant N170Q of *S. aureus* PC1 β-lactamase has been produced and its structure determined showing that the water molecule site is blocked by the glutamine side chain (Zawadzke *et al.*, 1996). Consequently, the enzyme can be acylated by substrates, but deacylation is impaired. These data are in conflict with the proposal of Gibson *et al.* (1990) and Lamotte-Brasseur *et al.* (1991), who proposed that Glu166 acts as the general base during both the acylation and deacylation steps, with the proton first being transferred from Ser70 to the carboxylate group via the bridging water molecule.

An interesting feature of the structure of the *S. aureus* PC1 β-lactamase is the presence of a non-proline *cis* peptide bond between Glu166 and Ile167 (Herzberg, 1991). Non-proline *cis* peptide bonds are rare in proteins of known structure. This is not surprising because there is an approximately 2.8 kcal/mol energy difference between the *cis* and *trans* conformations of a non-proline peptide bond as revealed by NMR experiments (Drakenberg *et al.*, 1972), and the *cis* conformation may be further destabilized by longer range interactions, resulting in an expected frequency of occurrence of about 0.1% in the denatured state (Ramachandran & Mitra, 1976). Analysis of the few crystallographically observed non-proline *cis* peptides in proteins revealed that they are associated with active sites (Herzberg & Moult, 1991b). The importance of the *cis* peptide bond for the *S. aureus* PC1 β-lactamase function is obvious in light of the crucial role of the carboxylate group of Glu166. The *cis* peptide bond is located on an Ω-loop encompassing residues 163−178, a structural unit which is not well packed against the rest of the molecule, with several intervening internal water molecules (Herzberg & Moult, 1987; Herzberg, 1991). In addition to the coordinates of the *S. aureus* β-lactamase [Brookhaven Protein Data Bank (PDB) entry code 3BLM], the coordinates of two other class A β-lactamases are available in the PDB, those of the enzymes from *B. licheniformis* and from *E. coli* (entry codes 4BLM and 1BLT, respectively). The latter two molecules contain a proline *cis* peptide, an energetically more favorable arrangement, and in addition, their respective Ω-loops are more efficiently packed against the rest of the structure with no internal water molecules. One would expect that the non-proline *cis* peptide and the inefficient packing that differentiate the *S. aureus* enzyme from its two counterparts are features that could

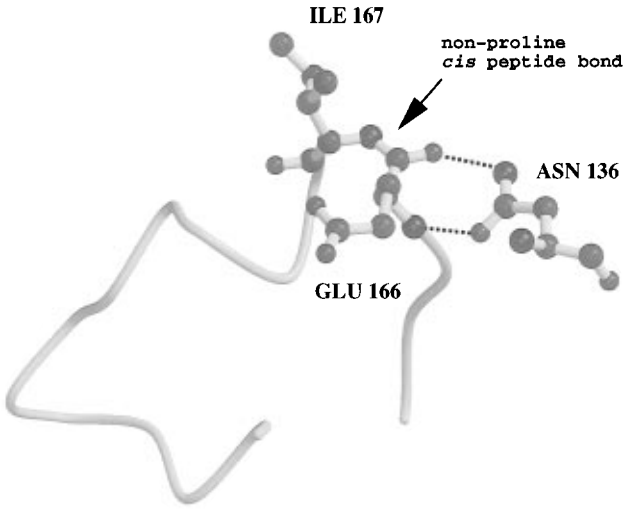

FIGURE 1: Ω-Loop region of β-lactamase. The trace of α-carbon atoms is highlighted in yellow except for residues 166 and 167 which are shown in an all-atom representation. The atoms' color scheme is as follows: gray, carbon; red, oxygen; and blue, nitrogen. The electrostatic interaction of the side chain of Asn136 with the main chain atoms of Glu166 is shown in dashed lines.

contribute to a larger conformational flexibility of the Ω-loop, even though that region is well-ordered in the crystal structure. This hypothesis is supported by the report that the *E. coli* TEM-1 mutant enzyme P167T is less stable compared with the wild-type enzyme (Vanhove *et al.*, 1996). Further, guanidine hydrochloride unfolding experiments of the TEM-1 wild-type and mutant proteins have been interpreted in terms of the *trans* to *cis* isomerization of the non-proline peptide bond comprising the rate-limiting step in the folding process (Vanhove *et al.*, 1996).

The carboxylate group of Glu166 forms electrostatic interactions with the side chains of Lys73 and Asn170. These are relevant to function and, in addition, may help maintain the structural integrity of the loop. Only three other side chains form Ω-loop-associated interactions which are conserved in all class A β-lactamases of known structure. A pair of side chains flanking the loop (Arg164 and Asp179) form a salt bridge. Elimination of this salt bridge by the D179N mutation of P54 β-lactamase has a profound functional and structural impact. The hydrolytic activity is much reduced, and folding experiments indicated that the molecule is trapped in a conformational state corresponding to a late step of the folding pathway as assessed by urea gradient gels (Craig *et al.*, 1985). The crystal structure of P54 β-lactamase revealed disorder in the Ω-loop region, including that of Glu166, and stopped-flow kinetics showed that the mutant enzyme is deacylation-impaired (Herzberg *et al.*, 1991).

The third invariant residue associated with the Ω-loop is Asn136. Its side chain amido group interacts with both the main chain amide and the carbonyl groups of Glu166, assisting in the precise positioning of the *cis* peptide bond (Figure 1). This study investigates this interaction. The mutant enzyme N136A has been produced, and the impact of the elimination of the side chain amido group on the activity and stability of the enzyme has been analyzed. The results provide further support for the proposal that the non-proline *cis* peptide contributes to the marginal stability of the Ω-loop. In addition, a surprising altered substrate recognition pattern of the mutant enzyme is revealed. Unlike the native *S. aureus* β-lactamase, it forms an acyl enzyme

with cephalosporins, including third-generation cephalosporins, but not with penicillins.

MATERIALS AND METHODS

Mutagenesis, Expression, and Protein Purification. N136A β-lactamase from *S. aureus* PC1 was cloned, expressed in *E. coli* TG1, and purified following a protocol similar to that in Zawadzke *et al.* (1995), with modifications described in Zawadzke *et al.* (1996). The four-primer overlap-extension method (Ho *et al.*, 1989) was used to produce the single-site mutant from the β-lactamase gene on pTS32 (*bla*Z). The two external primers were oligonucleotides LZBAMHI (5′-GTCCGGCGTAGAGGATCCGGAATTCT-CATG-3′) and TSHIND3 (5′-ATCAGTTTTTGATAT-CAAGCTTATACATGTCAACG-3′), and their respective internal mutagenic primers were oligonucleotides SBN136A1 (5′-AATACAGCAAAC**GCA**AAAATTATAA-3′) and SBN136A2 (5′-TTTATAATTTTGT**CGT**TTGCTGTATT-3′), which substitute an alanine for an asparagine (the changes relative to the wild-type enzyme are shown in bold letters). The single-site DNA mutant *bla*Z gene was then resequenced using double-strand plasmid DNA with the Sequenase Quick-Denature Plasmid DNA sequencing kit (U. S. Biochemicals, Cleveland, OH). The engineered gene includes an additional N-terminal methionine compared with the native amino acid sequence.

Protein concentrations were estimated from the absorbance of solutions at 280 nm by using the value for ϵ_{280} of 19 500 M^{-1} cm^{-1} (Carrey & Pain, 1978). For storage, the proteins were kept at 4 °C in solutions containing 60% saturated ammonium sulfate. This is essential for the N136A enzyme because this mutant is more susceptible to residual proteolytic activity compared with the wild-type enzyme. In the absence of ammonium sulfate, the mutant enzyme is completely degraded within a few days. Ammonium sulfate was removed by alternating steps of dilution and concentration in the centrifuge, using a Centricon 10 membrane.

Enzyme Kinetics. All kinetic assays were performed at 25 °C in 0.1 M potassium phosphate buffer at pH 6.8 on a Hewlett-Packard 8452A diode array spectrophotometer. The data were analyzed using the computer package SigmaPlot (Jandel Scientific). Nitrocefin was purchased from Unipath (Ogdensburg, NY). 6-β-[(Furylacryloyl)amido]penicillanic acid triethylamine salt (FAP) was purchased from Calbiochem (La Jolla, CA). Other β-lactam antibiotics were purchased from Sigma (St. Louis, MO). Hydrolysis of the chromogenic cephalosporin, nitrocefin, was monitored by the increase in absorbance at 500 nm ($\Delta\epsilon_{500} = 15\ 900$ M^{-1} cm^{-1} for the difference in absorption between the intact and hydrolyzed compound). The hydrolysis of other substrates was monitored by loss of absorbance as follows: benzylpenicillin ($\Delta\epsilon_{232} = 940$ M^{-1} cm^{-1}), methicillin ($\Delta\epsilon_{235} = 960$ M^{-1} cm^{-1}), ampicillin ($\Delta\epsilon_{235} = 820$ M^{-1} cm^{-1}), cloxacillin ($\Delta\epsilon_{260} = 1060$ M^{-1} cm^{-1}), oxacillin ($\Delta\epsilon_{260} = 1010$ M^{-1} cm^{-1}), FAP ($\Delta\epsilon_{344} = 1330$ M^{-1} cm^{-1}), cephaloridine ($\Delta\epsilon_{260} = 10\ 700$ M^{-1} cm^{-1}), cefotaxime ($\Delta\epsilon_{262} = 7250$ M^{-1} cm^{-1}), and ceftazidime ($\Delta\epsilon_{260} = 10\ 200$ M^{-1} cm^{-1}). These extiction coefficient values were determined previously (Ellerby *et al.*, 1990; Zawadzke *et al.*, 1995). In addition, the enzyme was assayed with the acyl-D-Ala-D-Ala analogue acyclic depsipeptide *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid, which is hydrolyzed by the wild-type enzyme (Govardhan & Pratt, 1987). The compound was provided by R. Pratt (Wesleyan University). Its hydrolysis was monitored at 290 nm ($\Delta\epsilon_{290} = 2290$ M^{-1} cm^{-1}, as determined in this work).

Preincubation kinetic experiments were carried out according to the following protocol. The mutant enzyme was first incubated with an approximately 2-fold excess of the substrate for a period of 90 s. The β-lactam compounds that were preincubated with the enzyme were benzylpenicillin, ampicillin, methicillin, cloxacillin, oxacillin, FAP, cephaloridine, cefotaxime, and ceftazidime. Each mixture was added to a cuvette containing an approximately 80-fold excess of nitrocefin over enzyme, and the nitrocefin hydrolysis was monitored in the usual manner.

Mass Spectrometry. Electrospray mass spectra were obtained on a JEOL SX102 mass spectrometer equipped with an Analytica electrospray source and on a Finnigan TSQ700 electrospray mass spectrometer. Samples were run in one of the following solutions: (a) 10 mM ammonium acetate; (b) 10 mM ammonium acetate with an equal volume addition of hexafluoro-2-propanol; (c) sample b with the addition of 5% acetic acid to hexafluoro-2-propanol (1:1); (d) desalted solution with an equal volume of hexafluoro-2-propanol; (e) sample d with the addition of 5% acetic acid to hexafluoro-2-propanol. The needle voltage was maintained at approximately 5 kV on the Analytica source and 4.5 kV on the Finnigan. In all cases, the spectra were deconvoluted using the standard software to produce mass (charge-independent) spectra. MALDI spectra were obtained on a Kratos MALDI III mass spectrometer operated in the linear mode at an accelerating voltage of 22 kV, with samples desorbed from a sinapinic acid matrix (3,5-dimethoxy-4-hydroxycinnamic acid) using a nitrogen (355 nm) laser. In preparation for mass spectrometry, the ammonium sulfate- and phosphate buffer-containing protein sample was dialyzed against 10 mM ammonium acetate at pH 7. The acyl enzyme complexes were formed by adding a 2-fold molar excess of β-lactams to the mutant enzyme. The mass spectrometry analysis was carried out over a period of 7 days, yet the acyl enzyme complexes remained intact.

NMR Spectroscopy. One-dimensional proton NMR spectra were acquired on a Bruker AMX-500 spectrometer at 25 °C. A 90% H_2O/10% D_2O solution containing 240 and 250 μM protein for the wild-type and mutant β-lactamases, respectively, 150 mM ammonium sulfate, and 90 mM potassium phosphate at pH 6.8 was prepared. Solvent suppression was achieved through weak presaturation at the water frequency. A spectral width of 6024 Hz was used with 2048 complex points. No resolution enhancement or baseline correction was used in processing the spectra.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) measurements were performed with a Hart 7707 DSC heat conduction scanning microcalorimeter. Half-milliliter samples of wild-type and mutant β-lactamases containing 2.5 mg of protein in a solution of 2.5 M ammonium sulfate and 0.1 M potassium phosphate at pH 6.8 were used. A scan rate of 15 K h^{-1} was applied. To determine any dependence of the parameters on scan rate, scans were also performed at 30 K h^{-1}. A least-squares fit of the two-state transition model $P_{folded} \leftrightarrow P_{unfolded}$ to the transition peak data was performed by the EXAM program (Kirchoff, 1993). The program utilizes a sigmoidal baseline to yield a van't Hoff enthalpy (ΔH_v) and transition temperature (T_m, the temperature at half of the peak area) and the

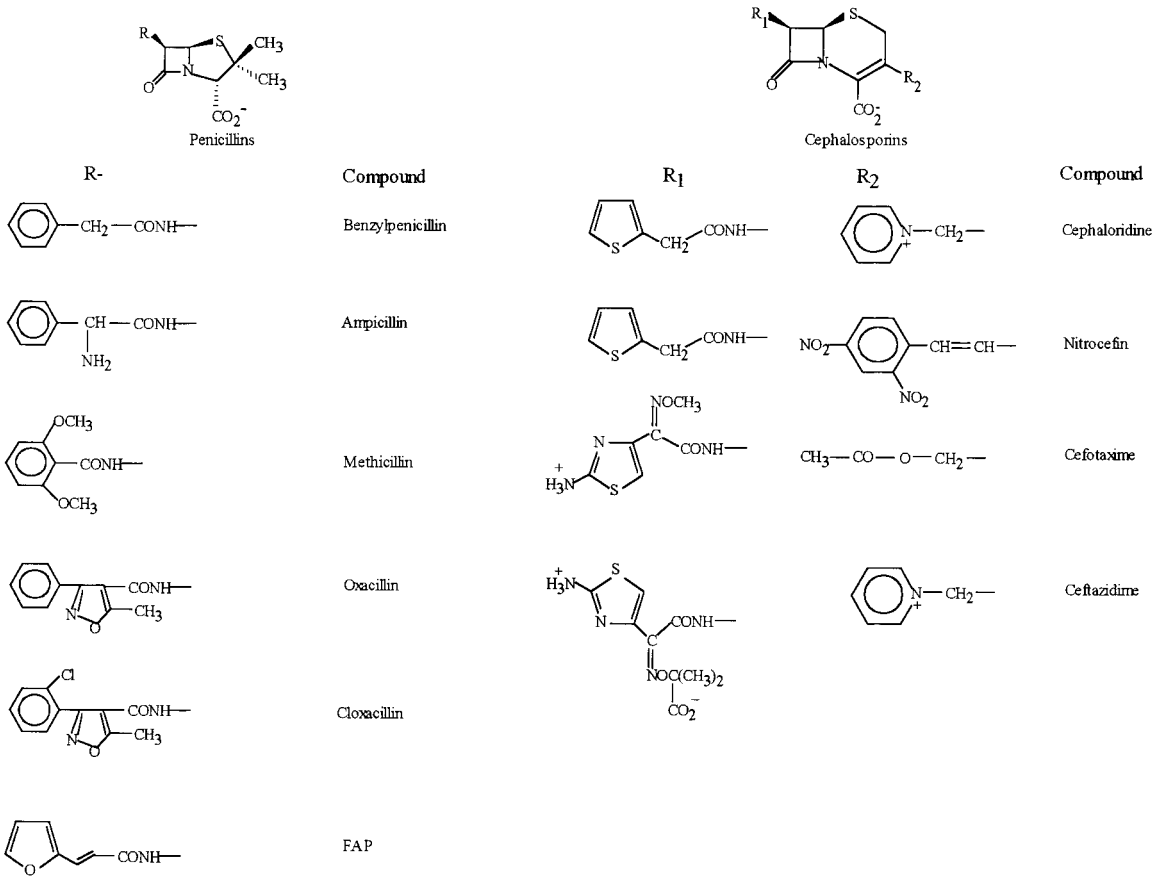

FIGURE 2: Scheme of the β-lactam antibiotics selected for this study. FAP is an abbreviation for 6-β-[(furylacryloyl)amido]penicillanic acid.

calorimetric enthalpy (ΔH_c, the transition peak area divided by the number of moles of protein in the cell). The ratio of $\Delta H_c/\Delta H_v$ yields the cooperativity of the transition.

Circular Dichroism. Circular dichroism (CD) spectra were measured with a Jasco 720 spectropolarimeter using a water-jacketed cylindrical cell with a path length of 1.0 mm. Temperature control was provided by a Neslab RTE-110 circulating water bath interfaced with a MTP-6 temperature programmer. All experiments were done in the presence of 0.1 M potassium phosphate buffer at pH 6.8.

Far-UV CD spectra of the wild-type and N136A mutant β-lactamases were scanned between 200 and 250 nm at 25 °C. Three scans were averaged. The protein samples contained 0.8 M ammonium sulfate. Unfolding transitions were monitored at 222 nm, varying the temperature from 0 to 90 °C at a rate of 1 °C/min. After reaching 90 °C, the system was cooled to its initial temperature of 0 °C, and the spectra were measured again to assess whether refolding was complete.

Urea denaturation and renaturation experiments were performed with wild-type and mutant protein samples in the presence of 80 mM ammonium sulfate and 0.1 M potassium phosphate buffer at pH 6.8. Samples containing enzyme and urea were prepared, with the urea concentration varying from 0 to 6.0 M and the enzyme concentration at 30 μM. After the fact that incubation for 1 h is required to reach equilibrium between the folded and unfolded species was established, the mixtures were incubated at 25 °C for 2 h and the CD spectra were measured, monitoring the ellipticity at 222 nm. For the refolding reaction, each of the previously equilibrated urea/enzyme mixtures was diluted into phosphate buffer (pH 6.8) to obtain the closest lower urea concentration value used in the unfolding experiment. The CD spectra were then collected, and the ellipticity was monitored at 222 nm. The analysis included the appropriate correction factors for dilutions.

Following the same protocol, the denaturation and renaturation experiments were repeated with the N136A β-lactamase, incubating the enzyme with 100 μM cephaloridine for a period of 10 min prior to the addition of urea. The concentrations of urea ranged from 0 to 5.5 M.

The kinetics of refolding of the N136A mutant enzyme was determined by incubating the protein solution in 5.5 M urea for a period of 2 h and diluting the enzyme/urea mixture with potassium phosphate buffer (pH 6.8) to a final urea concentration of 0.08 M. Aliquots were taken after 1, 2, and 5 min and in increments of 5 min thereafter and assayed for activity with nitrocefin. The final concentration of the mutant enzyme in the assay was 0.7 μM, while the concentration of nitrocefin was 100 μM. Rates were measured over 90 s for the first time point and for 150 s for the remaining points. The catalytic rates, k_{cat}, were analyzed as a function of elapsed time for refolding.

RESULTS

Enzyme Activity. With the series of substrates used in this study (Figure 2), the N136A mutant β-lactamase hydrolyzes only nitrocefin and the depsipeptide *m*-[[(phenylacetyl)-glycyl]oxy]benzoic acid. Hydrolysis of all other substrates were undetectable. With nitrocefin, the progress curve of hydrolysis exhibits a fast initial phase followed by a slower

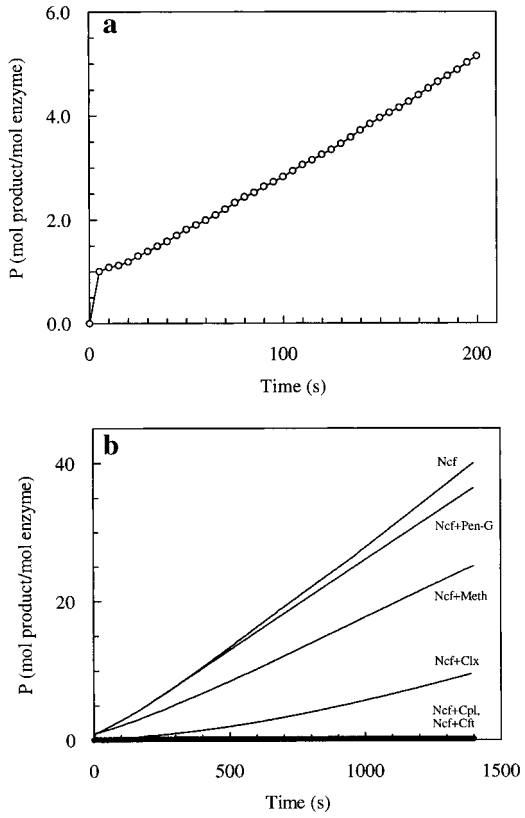

FIGURE 3: Kinetics of N136A β-lactamase. (a) Progress curve of nitrocefin hydrolysis by N136A β-lactamase. The concentrations of the enzyme and nitrocefin were 1.4 and 100 μM, respectively. The circles represent individual data points. (b) Hydrolysis of nitrocefin (Ncf) by N136A β-lactamase after incubation with various β-lactam antibiotics. The preincubation period was 90 s. The concentrations of the mutant enzyme, preincubated β-lactam, and nitrocefin were 1.3, 2.6, and 100 μM, respectively. Representative members of different types of progress curves are shown: benzylpenicillin (Pen-G), methicillin (Meth), cloxacillin (Clx), cephaloridine (Cpl), and ceftazidime (Cft). See Table 1 for data on the complete set used.

phase (Figure 3a). The slow phase has a slight lag before reaching the steady state, whereas no lag occurs with the wild-type enzyme (Herzberg *et al.*, 1991; Zawadzke *et al.*, 1995). The amplitude of the initial burst corresponds to a stoichiometry of 1 mol of product per mole enzyme. Note that the absorption of the acyl enzyme is similar to that of hydrolyzed nitrocefin and therefore the stoichiometric burst represents the formation of an acyl enzyme rather than a complete hydrolytic cycle. The slow phase was analyzed in terms of the general integrated equation

$$P = v_s t - (v_s - v_i)(1 - e^{-kt})/k \qquad (1)$$

where P is the concentration of the product at time t, v_i is the initial velocity, v_s is the steady state velocity (k_{cat}), and k is the rate constant characterizing the change from the initial to the steady state rates.

The k_{cat} derived from the slower phase was 0.0202 s^{-1} (Table 1). The K_m for the steady state phase was less than 1 μM and could not be determined accurately. By comparison, the k_{cat} value for nitrocefin hydrolysis by the wild-type enzyme was 14.0 s^{-1}, as was redetermined in the current study, and in agreement with the previously reported value for native β-lactamase of 11.4 s^{-1} (Herzberg *et al.*, 1991). The K_m value for the native enzyme is 7 μM, higher than the value for the N136A mutant enzyme.

Table 1: Nitrocefin Hydrolysis Rates by N136A β-Lactamase Following Incubation with Various β-Lactam Antibiotics[a]

preincubated β-lactam	$v_i \times 10^2$ (s^{-1})	$v_s \times 10^2$ (s^{-1})	$k \times 10^2$ (s^{-1})	burst
none	1.19 ± 0.01	2.02 ± 0.02	0.60 ± 0.02	1
none, 1.9 M $(NH_4)_2SO_4$	1.35 ± 0.01	3.69 ± 0.02	0.28 ± 0.01	1
benzylpenicillin	1.00 ± 0.03	1.97 ± 0.02	1.30 ± 0.04	1
ampicillin	1.07 ± 0.06	1.93 ± 0.04	1.31 ± 0.07	1
methicillin	0.70 ± 0.01	1.69 ± 0.01	0.39 ± 0.03	1
oxacillin	0.18 ± 0.01	1.48 ± 0.62	0.05 ± 0.01	−
cloxacillin	0.18 ± 0.01	1.41 ± 0.75	0.06 ± 0.01	−
cephaloridine	0	0	0	−
ceftazidime	0	0	0	−
cefotaxime	0	0	0	−

[a] The errors associated with the fit to the general integrated equation are provided.

Nitrocefin hydrolysis by the N136A β-lactamase was followed in the presence of 1.9 M ammonium sulfate, to assess whether the burst kinetics is due to the purely defective deacylation apparatus or whether it is associated with substrate-induced progressive inactivation, also termed branched pathway (Waley, 1991). The rationale is that, if there is partitioning between active and inactive acyl enzyme forms, a stabilizing agent such as ammonium sulfate (Michinson & Pain, 1985) would decrease the conversion into the inactive acyl enzyme and the size of the burst would increase (Escobar *et al.*, 1994; Zawadzke *et al.*, 1996). The burst amplitude remained 1 at high ammonium sulfate concentrations, indicative of simple impairment of the deacylation machinery rather than progressive inactivation. The lag was still observed (data not shown). v_i increased slightly to 0.0135 s^{-1}, and v_s increased to 0.0369 s^{-1} (Table 1). That ammonium sulfate accelerates the catalytic rates of the *S. aureus* β-lactamase is not unusual. This was previously reported for both the native enzyme and the deacylation-impaired P54 mutant β-lactamase (Herzberg *et al.*, 1991).

The hydrolysis rate constant of the depsipeptide by the N136A mutant enzyme was 0.118 s^{-1}, somewhat higher than the rate constant of 0.077 s^{-1} for the wild-type enzyme. The latter value agrees well with the rate constant reported previously (Govardhan & Pratt, 1987).

The substrates that are not hydrolyzed by N136A β-lactamase were further investigated for complex formation with the mutant enzyme. Each substrate was mixed with the enzyme, and after a preincubation period of 90 s, the kinetics of nitrocefin hydrolysis was monitored. The data show that, following preincubation with the traditional penicillins, benzylpenicillin and ampicillin, the activity toward nitrocefin is essentially unaltered, whereas other substrates do affect nitrocefin hydrolysis (Table 1, Figure 3b). Preincubation with penicillins with bulky side chain substituents on the β-lactam ring (methicillin, cloxacillin, and oxacillin) produced the following effects. Nitrocefin hydrolysis rates were reduced, with methicillin having less impact than cloxacillin and oxacillin; the lag of the slow phase was longer, again with the least effect observed with methicillin, and the stoichiometric fast burst was eliminated after preincubation with cloxacillin and oxacillin but not with methicillin.

Preincubation with cephaloridine and with the third-generation cephalosporins, cefotaxime and ceftazidime, resulted in the most dramatic changes. Both the burst and the hydrolytic activity toward nitrocefin were eliminated.

Note that the ceftazidime preincubation experiment was repeated with the wild-type enzyme because it also does not hydrolyze this compound. In contrast to the mutant enzyme, the kinetics of nitrocefin hydrolysis by the wild-type β-lactamase was unaltered, indicating that it does not interact tightly with ceftazidime.

Mass Spectrometry of the Enzyme/Cephalosporin Complexes. In order to determine the nature and stoichiometry of the addition products, the free N136A β-lactamase and the adducts formed with cephaloridine and cefotaxime were examined by mass spectrometry. MALDI spectra were obtained for the free mutant protein and the adducts. These showed an addition of only one molecule of antibiotic to the protein. No dissociation (as would be expected if a noncovalent adduct were formed) of the protein/antibiotic complex was observed. Previous studies of the hydrolysis of cephalosporins by β-lactamase from *S. aureus* PC1 concluded that the 3′-substituents (R2 in Figure 2) of cephalosporins may be eliminated prior to deacylation (Faraci & Pratt, 1985). The mass shifts predicted for the cephaloridine and cefotaxime if no elimination of the 3′-substituents occur are 415 and 455, respectively. After elimination, the predicted mass shifts are 336 and 395, respectively. For each antibiotic, MALDI gave varying answers that were both above and below the predicted masses without the elimination of the 3′-group. This was attributed to adduct formation with the ammonium salts as the tops of the peaks were always multiplets. The protein samples were desalted by precipitation with acetone and analyzed by electrospray mass spectrometry on an Analytica source in a 5% acetic acid/hexafluoro-2-propanol (1:1) solution. Under these denaturing conditions, a noncovalently bound substrate would dissociate whereas a covalently bound adduct can be detected. The free protein was measured at 28 881.7 (calculated mass of 28 882.4), and the two adducts were measured at 29 176.2 (if the 3′-substituent were eliminated, the calculated mass of the cephaloridine/protein complex would be 29 218.4) and 29 228.9 (the mass of a cefotaxime-bound enzyme would be 29 277.4 if the 3′-substituent is eliminated). In each case, the results obtained were approximately 44 mass units less than expected, and this was attributed to decarboxylation adjacent to the 3′-group that occurred in the mass spectrometer. In order to try to prevent decarboxylation, the samples were run at pH 7 in the absence of acetic acid, but the same result was obtained. The result for cefotaxime is shown in Figure 4. As none of the samples (protein and complexes) gave spectra when sprayed from the 10 mM ammonium acetate solution alone, the samples were desorbed from a 1:1 solution of 10 mM ammonium acetate and hexafluoro-2-propanol. In this case, variable spectra were obtained. In most, decarboxylation was complete or almost complete, but in some, especially on the Finnigan source, an almost totally nondecarboxylation product spectrum was obtained for the cephaloridine adduct (two runs, 29 217.5 and 29 228.0). Therefore, decarboxylation may be a feature of the electrospray ionization process. It did not seem to occur in the MALDI experiment, but these were considered less accurate because of the involvement of peak multiplets.

Structural Information. Figure 5 shows far-UV CD spectra of the wild-type and the N136A mutant enzymes. The mutant enzyme exhibits a considerable amount of regular secondary structure, although the signal is somewhat lower than that for the wild-type protein. The one-dimensional ^{1}H

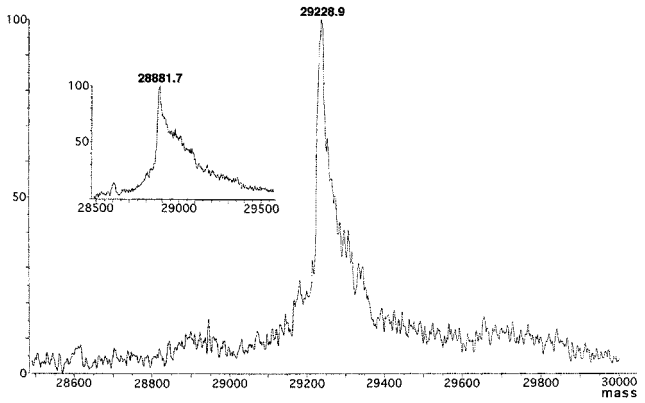

FIGURE 4: Mass spectrum of the adduct formed between cefotaxime and N136A β-lactamase. The protein solution was desalted and mixed with an equal volume of hexafluoro-2-propanol. The predicted weight of the adduct was 29 277.4, but decarboxylation was observed to occur in most electrospray runs. The single peak indicates that only a 1:1 antibiotic/protein adduct is formed. The inset shows the free protein (calculated weight of 28 882.4).

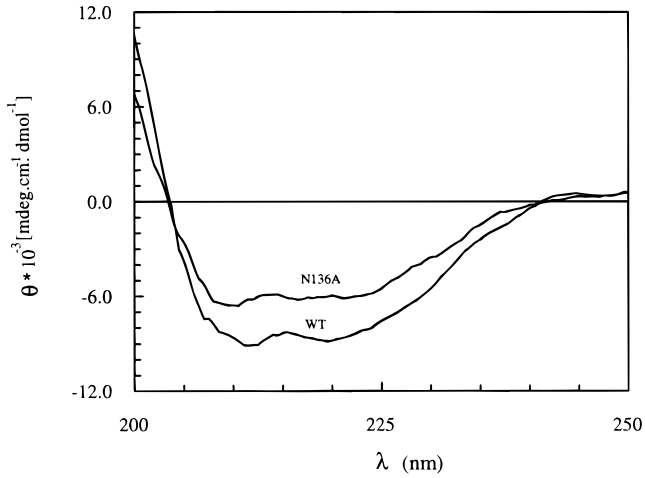

FIGURE 5: Far-UV circular dichroism spectra of the wild-type and N136A β-lactamases at 25 °C. For each, the mean residue ellipticity is plotted as a function of wavelength. The concentration of each protein was 30 μM, and the solutions contained 0.8 M ammonium sulfate and 0.1 M potassium phosphate at pH 6.8.

NMR spectrum of the mutant β-lactamase shows that as with the wild-type enzyme the spectrum of the mutant enzyme is well-dispersed (Figure 6). The CD and NMR spectra, together with the observation that the mutant enzyme undergoes acylation with some substrates, indicate that the molecule adopts a unique global fold.

Thermal Stability. The DSC of wild-type β-lactamase in an ammonium sulfate solution revealed a single transition peak closely fitted to the two-state $P_{folded} \leftrightarrow P_{unfolded}$ transition model with a T_m of 77.0 ± 1.0 °C. The transition peak did not re-appear upon a subsequent cooling and re-heating of the solution, indicating that the transition was irreversible. An irreversible transition was also reported by Rahil and Pratt (1994), who using the protein absorption at 280 nm observed a T_m value of 41.9 °C and a broader transition. The sharper transition at higher temperature observed in the current study is attributed to the high ammonium sulfate concentration used.

From the fit of the two-state model, the ΔH_c is 134 ± 1 kcal/mol and the ΔH_v is 304 ± 17 kcal/mol, yielding a cooperativity of 0.5. Since the same result was obtained at twice the scan rate, it was assumed that the irreversible

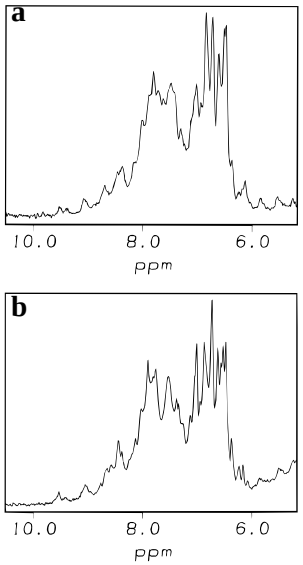

FIGURE 6: One-dimensional proton NMR spectra of (a) wild-type and (b) N136A β-lactamases showing the amide and aromatic proton regions.

transition could be treated as a thermodynamic two-state transition. Thus, the discrepancy between ΔH_c and ΔH_v implies that, under the experimental conditions, wild-type β-lactamase unfolds as a dimer at its thermal denaturation temperature.

The DSC of the N136A mutant β-lactamase yielded the same values of ΔH_c and ΔH_v as calculated for the wild-type enzyme but a substantially lower T_m of 64.0 ± 1.0 °C, indicating that the mutant enzyme is conformationally less stable in solution than the wild-type enzyme.

A similar trend for T_m values was seen in the CD in the presence of 0.8 M ammonium sulfate. The wild-type protein unfolded at 74 ± 1 °C and the N136A mutant protein at 68 ± 1 °C. As with the DSC, the transition fits a two-state model and is irreversible under the conditions of the experiment. When the solution was slowly cooled to 0 °C, the CD signal did not recover to that observed prior to the heating.

Urea Denaturation. The unfolding of β-lactamase from *S. aureus* PC1 in urea is reversible, and the rate constant is independent of enzyme concentration (Mitchinson & Pain, 1985; Craig *et al.*, 1985). The comparison between the unfolding profiles of the wild-type and the N136A mutant enzymes, followed by CD, reveals that the midpoint transition for the mutant enzyme occurs at a considerably lower urea concentration (Figure 7). Since the unfolding is reversible, the data were analyzed by a two-state equilibrium:

$$P_{folded} \overset{K_D}{\Leftrightarrow} P_{unfolded}$$

The apparent equilibrium constant K_D was calculated following the analysis of Santoro and Bolen (1988), using nonlinear least squares to fit the data to the exponential equation that can be reduced to

$$f = 1 - 1/[1 + e^{(m_1 - m_2 x)}] \quad (2)$$

where f is the fraction of unfolded protein estimated from the loss of ellipticity at 222 nm at each urea concentration, $m_1 = -\ln K_D$, $m_2 = \Delta(-\ln K_D)/\Delta[\text{urea}]$, and $x = [\text{urea}]$.

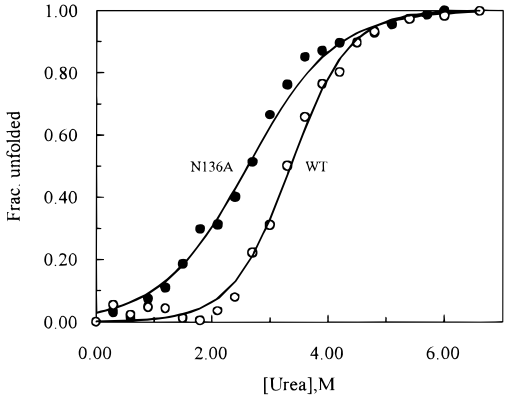

FIGURE 7: Apparent unfolded fractions of wild-type (open circles) and N136A (filled circles) β-lactamases as a function of urea concentration. The fraction of unfolded protein was calculated from the CD signal at 222 nm. The concentration of each enzyme was 30 μM. Note that the data points at 0.6 M urea for the mutant and wild-type enzymes overlap.

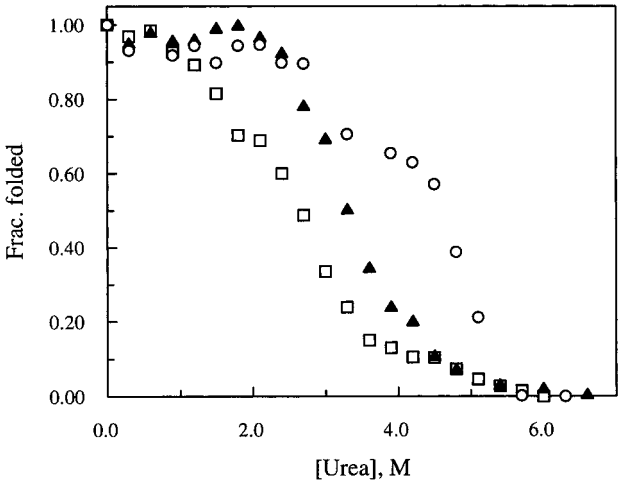

FIGURE 8: Unfolding of the wild-type β-lactamase (filled triangles), N136A β-lactamase (open squares), and N136A β-lactamase plus cephaloridine (open circles) by urea. The fraction of unfolded protein was calculated from the CD signal at 222 nm. The final concentrations of the enzymes were 30 μM, and the concentration of cephaloridine was 100 μM.

K_D values for unfolding extrapolate to 883.3 and 38.5 in the absence of urea for the wild-type and N136A mutant enzymes, respectively. The free energy of folding, $\Delta G_{folding}$, at 25 °C is −4.0 kcal/mol for the wild-type protein and −2.1 kcal/mol for the mutant enzyme. The results for the wild-type protein are consistent with those reported by Mitchinson and Pain (1985).

To further investigate the enzyme−substrate interaction, the unfolding experiment was repeated for the N136A enzyme that was preincubated with cephaloridine. The data are shown in Figure 8, together with the unfolding data for the mutant enzyme alone and for the wild-type protein. Incubation with cephaloridine resulted in the increased stability of the mutant enzyme beyond the stability of the wild-type enzyme. Also, the transition curve exhibits a biphasic transition, which may indicate the existence of intermediates in the unfolding pathway. The data were fitted to two independent two-state unfolding processes according to the scheme

$$P_{folded} \overset{K_{D_1}}{\Leftrightarrow} P_{intermediate} \overset{K_{D_2}}{\Leftrightarrow} P_{unfolded}$$

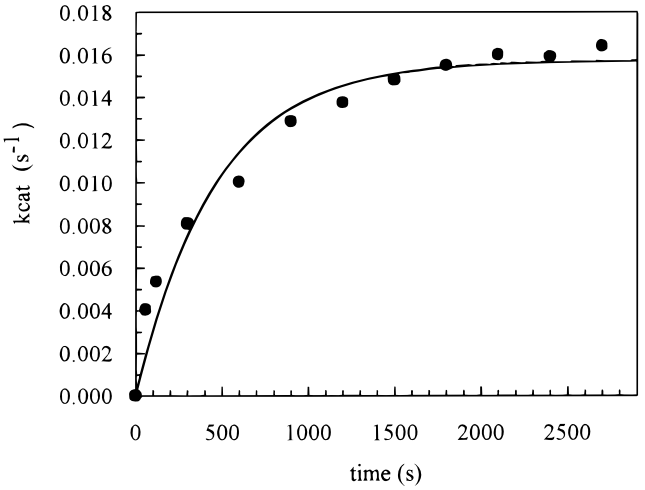

FIGURE 9: Refolding kinetics of urea-denatured N136A β-lactamase followed by the recovery of the nitrocefin hydrolysis rate. Enzyme samples were diluted into a final urea concentration of 0.08 M. Aliquots were taken after 1, 2, and 5 min and in increments of 5 min thereafter. The final concentration of the enzyme in the assays was 0.7 μM, and the concentration of nitrocefin was 100 μM.

where K_{D_1} and K_{D_2} are the apparent equilibrium constants for the two processes. K_{D_1} and K_{D_2} extrapolate to 3.83×10^3 and 3.70×10^5 in the absence of urea, respectively. The corresponding values of $\Delta G_{folding}$ for the processes $P_{folded} \leftrightarrow P_{intermediate}$ and $P_{intermediate} \leftrightarrow P_{unfolded}$ at 25 °C are -4.8 and -7.6 kcal/mol, respectively. The plateau region of the proposed folding intermediate is not well-defined. Thus, these values are not accurately determined and should be regarded only as estimates.

The kinetics of refolding was monitored by measuring the increase in the nitrocefin hydrolysis rate of urea-denatured samples after dilution into a urea-free solution for increasing time periods (Figure 9). The refolding of the wild-type enzyme occurs too rapidly to be monitored in this manner, but the refolding of the N136A mutant enzyme is sufficiently slow. The data were analyzed by a single-exponential function according to

$$k_{cat,t} = k_{cat,f}(1 - e^{-kt}) \quad (3)$$

where $k_{cat,t}$ is the rate constant of hydrolysis at time t after the dilution, $k_{cat,f}$ is the final hydrolytic rate constant, and k is the folding rate constant. The exponential fit yielded a k of 0.0022 ± 0.0003 s^{-1} and a $k_{cat,f}$ of 0.0157 ± 0.0005 s^{-1}. Note that only 80% of the catalytic rate was recovered. Partial recovery of activity of refolded *S. aureus* PC1 β-lactamase was previously reported (Mitchinson & Pain, 1985) and was attributed to partial adsorption and/or aggregation of unfolded enzyme.

DISCUSSION

Enzyme Activity. The three-dimensional atomic structure of the N136A mutant β-lactamase is unavailable because crystals suitable for X-ray work have not been obtained so far. Nevertheless, the CD and NMR data are consistent with a protein adopting a global folded state. Also, the measurable enzymatic activity toward nitrocefin implies that the spatial arrangement of at least part of the catalytic apparatus is preserved; hence, the structure integrity is also preserved.

Using mechanism-based inhibitors, it has been established that the hydrolysis of β-lactams by the class A β-lactamases involves an acyl enzyme intermediate (Knott-Hunziker *et al.*, 1979; Cartwright & Coulson, 1980; Cohen & Pratt, 1980; Fisher *et al.*, 1980). The simplest mechanism that accounts for the acyl enzyme intermediate is shown in Scheme 1:

Scheme 1

$$E + S \overset{K_S}{\leftrightarrow} ES \xrightarrow{k_2} EC \xrightarrow{k_3} E + P$$

where the enzyme, substrate, and product are depicted by E, S, and P, respectively, ES denotes the Michaelis complex, and EC denotes the acyl enzyme complex. The acylation step is considered irreversible when the deacylation step is fast, but with deacylation-impaired enzymes, reversibility of the acylation may need to be considered.

With the exception of nitrocefin, the mutation N136A abolished the ability of the enzyme to hydrolyze β-lactam compounds. For nitrocefin, a fast initial burst corresponds to the appearance of a stoichiometric amount of acyl enzyme. The following slower phase reflects the incompetence of the mutant enzyme to catalyze the deacylation step (Figure 3a). The slow phase exhibits a short lag, suggestive of a substrate-induced activation. However, this is a subtle effect, and for for the sake of simplicity, the kinetics of nitrocefin hydrolysis by this mutant enzyme will be treated according to Scheme 1. The magnitude of the burst is not affected by the addition of ammonium sulfate, ruling out the kinetic scheme of a branched pathway mechanism corresponding to substrate-induced progressive inactivation (Waley, 1991; Escobar *et al.*, 1994; Zawadzke *et al.*, 1996).

The burst and the steady state hydrolytic rate for nitrocefin are similar to those measured with two seemingly very different mutant β-lactamases: D179N (termed P54) (Herzberg *et al.*, 1991) and N170Q (Zawadzke *et al.*, 1996). With the first mutant enzyme, a salt bridge at the bottom of the Ω-loop has been eliminated. Part of the loop becomes disordered, including Glu166, the residue that enhances the nucleophilicity of the hydrolytic water molecule. Consequently, deacylation is impaired. In the second mutant enzyme, N170Q, the site of the hydrolytic water molecule has been blocked by the glutamine, also leading to impaired deacylation. The N136A mutation was engineered to destabilize the Ω-loop by removing key electrostatic interactions between the amido group of Asn136 and the main chain atoms of Glu166, interactions that assist in fixing the position of the sterically strained non-proline *cis* peptide bond (Figure 1). The hypothesis was that these interactions are crucial to the structural integrity of the loop because only a few other interactions are formed with the rest of the molecule. Indeed, as with P54 β-lactamase, the N136A mutant enzyme is deacylation defective. In fact, the kinetic consequences of the N136A replacement are more severe than those of the D179N mutation of the P54 enzyme. Whereas the hydrolysis rate of benzylpenicillin by the P54 β-lactamase is substantially reduced, but is still measurable, the N136A enzyme does not hydrolyze any of the β-lactams that have been studied except for nitrocefin.

The P54 enzyme exhibits residual activity with a number of penicillins and cephalosporins (Herzberg *et al.*, 1991; O. Herzberg and A. F. W. Coulson, unpublished results). Therefore, the activity may perhaps be attributed to infrequent sampling of the native conformation of the Ω-loop in which Glu166 and its adjacent water molecule are positioned

appropriately for deacylation to occur. In contrast, the failure of the N136A mutant enzyme to undergo deacylation with any of the penicillins or cephalosporins except for nitrocefin indicates that the productive conformation of the Ω-loop is not sampled at all. If so, the low hydrolysis rate of nitrocefin and also of the depsipeptide may be attributed to alternative mechanisms which are unavailable for the rest of the β-lactam substrates. The nature of such mechanisms is yet unknown, but perhaps the *S. aureus* β-lactamase acyl enzyme complexes of these two compounds are chemically unstable, allowing a less nucleophilic water molecule to play the hydrolytic role.

A single acylation step is undetectable in assays using β-lactam antibiotics with low extinction coefficients measured at wavelengths where the protein absorbs as well. Therefore, the nitrocefin activity was used to indirectly assess whether these compounds bind to the N136A mutant enzyme. Nitrocefin was used previously as a reporter substrate for β-lactam compounds that inactivate β-lactamases (Galleni & Frère, 1988; Matagne *et al.*, 1990). In the current study, for those β-lactams that form stable acyl enzyme complexes, incubation of the compounds with the protein in close to stoichiometric amounts followed by assaying for nitrocefin activity should be manifested by elimination of nitrocefin hydrolysis. The caveat is that a very tight noncovalent complex may also produce the same kinetic effect. Performing the mass spectrometry under denaturing conditions distinguished between the two possibilities.

For the series of β-lactam antibiotics used, elimination of nitrocefin hydrolysis occurred with the traditional cephalosporin, cephaloridine, and with the third-generation cephalosporins, cefotaxime and ceftazidime (Figure 3b, Table 1). A control experiment with the wild-type enzyme showed that nitrocefin hydrolysis is unaffected by preincubation with ceftazidime, confirming that in that case ceftazidime does not form a stable acyl enzyme.

Both MALDI and electrospray mass spectrometry revealed adducts with a stoichiometry of 1:1 between the mutant protein and the antibiotic. Moreover, all experiments were carried out under denaturing conditions, when a noncovalent complex would dissociate. Therefore, they confirm the formation of stable acyl enzyme complexes between N136A β-lactamase and either first- or third-generation cephalosporins and rule out tight noncovalent complexes that do not undergo acylation. Clearly, the active site of N136A β-lactamase recognizes first- and third-generation cephalosporins rather than penicillins. The implication is that for acylation, the mutant enzyme exhibits altered specificity compared with the wild-type enzyme.

Structure−Activity Relationship. The structural rationale for the kinetic properties of N136A β-lactamase remains speculative in the absence of a crystal structure. Nevertheless, with the structure of the native and P54 enzymes in mind, we propose that, by eliminating the key interactions between the side chain of Asn136 and the main chain carbonyl and amide groups of Glu166, the *cis* peptide is destabilized and part or all of the Ω-loop unfolds. Without Glu166 in position to enhance the nucleophilicity of the hydrolytic water, the mutant enzyme is deacylation defective. Moreover, without the Ω-loop in its native conformation, other polypeptide segments within the active site may be perturbed, leading to altered specificity. For example, the edge β-strand comprising residues 234−239 could be affected because of its proximity to the Ω-loop. In the absence of the constraints imposed by the loop, the β-strand may shift such that the positions of the oxyanion hole and Lys234 relative to the catalytic Ser70 hydroxyl group may change. Two key groups of the β-lactam antibiotics anchor the compound to the oxyanion hole and to Lys234: the carbonyl oxygen atom of the β-lactam bond and the carboxyl group on the fused ring. These groups are oriented differently in penicillins and cephalosporins. Therefore, even a subtle change in the β-strand may lead to a new substrate preference (Herzberg & Moult, 1991a). The disorder of the Ω-loop may also account for the ability of the mutant enzyme to bind β-lactams with bulky side chains because the active site gully, where the side chain is accommodated, should be more open and perhaps more flexible.

Stability of N136A β-Lactamase. Both thermal denaturation and urea-induced denaturation show that the mutant enzyme is less stable than the wild-type enzyme. The 2 kcal/mol stability difference revealed by the urea denaturation experiments should not be attributed solely to the loss of two hydrogen bonds arising from the replacement of Asn136 by an alanine, because the expected structural changes are substantial. At least the following two changes are expected to be energetically favorable. First, the loss of the *cis* peptide bond would relieve steric strain. Second, disorder of the Ω-loop is entropically favorable. On the other hand, the loss of hydrophobic and electrostatic interactions between the loop and the remaining molecule is energetically unfavorable. In principle, there may also be a free energy effect on the unfolded state. It is the energy balance between all these terms that ultimately results in destabilization by 2 kcal/mol of the mutant protein compared with the wild-type protein.

The replacement of Asn136 by an alanine also slowed the folding process considerably compared with that of the wild-type enzyme. While wild-type protein refolding is much too fast to follow by recovery of enzymatic activity, the refolding of the mutant enzyme could be followed in this manner. If indeed the Ω-loop is disordered in the mutant enzyme, it is tempting to speculate why its integrity is important for the kinetics of folding. Because of its location at the interface of two domains, the loop may help accelerate the process of domain association. On the other hand, the inability of the loop to adopt a unique conformation because of the N136A replacement may increase the mobility of the polypeptide chain and interfere with folding.

Finally, in the presence of cephaloridine, a compound that forms a stable complex with the N136A β-lactamase, the stability of the mutant enzyme is enhanced beyond the stability of the wild-type enzyme (Figure 8). In addition, the folding curve is biphasic, indicative of an intermediate which is not seen with either the wild-type or the mutant enzymes alone. Binding is in a depression, at the interface between the two domains of the molecule. Favorable hydrophobic and electrostatic interactions would be formed, which may compensate for the loss of interactions due to disorder of the Ω-loop. Note that the stability of wild-type enzyme is not expected to increase in that manner because it hydrolyzes cephaloridine and does not form a stable complex. However, compounds that form stable complexes with the wild-type enzyme lead to a similar phenomenon, as has been reported previously for the β-lactamase from *S. aureus* PC1 in the presence of phosphonate inhibitors (Rahil & Pratt, 1994).

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REFERENCES

Abraham, E. P., & Chain, E. B. (1940) *Nature 146*, 837.
Adachi, H., Ohta, T., & Matsuzawa, H. (1991) *J. Biol. Chem. 266*, 3186−3191.
Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, Ser. B 289*, 321−331.
Ambler, R. P., Coulson, A. F. W., Frère, J.-M., Ghuysen, J.-M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., & Waley, S. G. (1991) *Biochem. J. 276*, 269−270.
Carrey, E. A., & Pain, R. H. (1978) *Biochim. Biophys. Acta 533*, 12−22.
Cartwright, S. J., & Coulson, A. F. W. (1980) *Philos. Trans. R. Soc. London, Ser. B 289*, 370−372.
Chen, C. C., Smith, T. J., Kapadia, G., Wäsch, S., Zawadzke, L. E., Coulson, A., & Herzberg, O. (1996) *Biochemistry 35*, 12251−12258.
Cohen, S. A., & Pratt, R. F. (1980) *Biochemistry 19*, 3996−4003.
Coulson, A. (1985) *Biotechnol. Genet. Eng. Rev. 3*, 219−253.
Craig, S., Hollecker, M., Creighton, T. E., & Pain, R. H. (1985) *J. Mol. Biol. 185*, 681−687.
Damblon, C., Raquet, X., Lian, L.-Y., Lamotte-Brasseur, J., Fonze, E., Charlier, P., Roberts, G. C. K., & Frère, J.-M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1747−1752.
Drakenberg, T., Dahlqvist, K.-I., & Forśen, S. (1972) *J. Phys. Chem. 76*, 2178−2183.
Ellerby, L. M., Escobar, W. A., Fink, A. L., Michinson, C., & Wells, J. A. (1990) *Biochemistry 29*, 5797−5806.
Escobar, W. A., Tan, A. K., & Fink, A. L. (1991) *Biochemistry 30*, 10783−10787.
Escobar, W. A., Tan, A. K., Lewis, E. R., & Fink, A. L. (1994) *Biochemistry 33*, 7619−7626.
Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903−910.
Fisher, J., Belasco, J. G., Kohsla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895−2901.
Frère, J.-M., & Joris, B. (1985) *CRC Crit. Rev. Microbiol. 11*, 299−396.
Galleni, M., & Frère, J.-M. (1988) *Biochem. J. 255*, 119−122.
Gibson, R. M., Christensen, H., & Waley, S .G. (1990) *Biochem. J. 272*, 613−619.
Govardhan, C. P., & Pratt, R. F. (1987) *Biochemistry 26*, 3385−3395.
Hamilton-Miller, J. M. T., & Smith, J. T., Eds. (1979) *Beta-Lactamases*, Academic Press, London.
Herzberg, O. (1991) *J. Mol. Biol. 217*, 701−719.
Herzberg, O., & Moult, J. (1987) *Science 236*, 694−701.
Herzberg, O., & Moult, J. (1991a) *Curr. Opin. Struct. Biol. 1*, 946−953.
Herzberg, O., & Moult, J. (1991b) *Proteins: Struct., Funct., Genet. 11*, 223−229.
Herzberg, O., Kapadia, G., Blanco, B., Smith, T., & Coulson, A. F. W. (1991) *Biochemistry 30*, 9503−9509.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51−59.
Jelsch, C., Lenfant, F., Masson, J. M., & Samama, J. P. (1992) *FEBS Lett. 299*, 135−142.
Jelsch, C., Mourey, L. F., Masson, J. M., & Samama, J. P. (1993) *Proteins 16*, 364−383.
Kirchoff, W. H. (1993) NIST Technical Note 1401, pp 1−103, U.S. Department of Commerce, Gaithersburg, MD.
Knott-Hunziker, V., Waley, S. G., Orlek, B. S., & Sammes, P. G. (1979) *FEBS Lett. 99*, 59−61.
Knowles, J. R. (1985) *Acc. Chem. Res. 18*, 97−102.
Knox, J. R., & Moews, P. C. (1991) *J. Mol. Biol. 220*, 435−455.
Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M., & Ghuysen, J.-M. (1991)*Biochem. J. 279*, 213−221.
Lobkovsky, E., Billing, E. M., Moews, P. C., Rahil, J., Pratt, R. F., & Knox, J. R. (1994) *Biochemistry 33*, 6762−6772.
Matagne, A., Misselyn-Bauduin, A.-M., Joris, B., Erpicum, T., Granier, B., & Frère, J.-M. (1990) *Biochem. J. 265*, 131−146.
Mitchinson, C., & Pain, R. H. (1985) *J. Mol. Biol. 184*, 331−342.
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frère, J.-M. (1990) *Proteins 7*, 156−171.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. 61*, 201−301.
Oefner, C., D'Arcy, A., Daly, J. J., Gubernato, K., Charnas, R. L., Heinze, I., Hubschwerlen, C., & Winkler, F. K. (1990) *Nature 343*, 284−288.
Rahil, J., & Pratt, R. F. (1994) *Biochemistry 33*, 116−125.
Ramachandran, G. N., & Mitra, A. K. (1976) *J. Mol. Biol. 107*, 85−92.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063−8068.
Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., & James, M. N. G. (1992) *Nature 359*, 700−705.
Vanhove, M., Raquet, X., Palzkill, T., Pain, R. H., & Frère, J.-M. (1996) *Proteins: Struct., Funct., Genet. 25*, 104−111.
Waley, S. G. (1991) *Biochem. J. 279*, 87−94.
Zawadzke, L. E., Smith, T., & Herzberg, O. (1995) *Protein Eng. 8*, 1275−1285.
Zawadzke, L. E., Chen, C. C. H., Banerjee, S., Li, Z., Wäsch, S., Kapadia, G., Moult, J., & Herzberg, O. (1996) *Biochemistry 35*, 16475−16482.

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