Site-directed mutagenesis of dicarboxylic acids near the active site of *Bacillus cereus* 5/B/6 β -lactamase II

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An amino acid residue functioning as a general base has been proposed to assist in the hydrolysis of β -lactam antibiotics by the zinc-containing *Bacillus cereus* β -lactamase II [Bicknell & Waley (1985) *Biochemistry* **24**, 6876–6887]. Oligonucleotide-directed mutagenesis of cloned *Bacillus cereus* 5/B/6 β -lactamase II was used in an 'in vivo' study to investigate the role of carboxy-group-containing amino acids near the active site of the enzyme. Substitution of asparagine for the wild-type aspartic acid residue at position 81 resulted in fully functional enzyme. An aspartic acid residue at position 90 is essential for β -lactamase II to confer any detectable ampicillin and cephalosporin C resistance to *Escherichia coli*. Conversion of Asp⁹⁰ into Asn⁹⁰ or Glu⁹⁰ lead to the synthesis of inactive enzyme, suggesting that the spatial position of the β -carboxy group of Asp⁹⁰ is critical for enzyme function.

INTRODUCTION

Bacillus cereus β -lactamase II is quite different in substrate specificity, cofactor requirement and heat-stability from the other two β -lactamases produced by this micro-organism. Unlike β -lactamase I and III, it hydrolyses a wide variety of β -lactam substrates, including cephalosporins, requires at least one zinc ion for activity, and retains significant activity after heating at 60 °C for 30 min [1].

The three-dimensional structure of the active site containing the residues constituting the primary zinc-binding site has been defined [2–4]. The primary zinc ligands consist of three histidine residues at positions 86, 88, and 210 and the only cysteine in the enzyme at position 168. The detailed mechanism of action of the enzyme and the residues critical for catalysis remain largely unknown. A number of possible mechanisms for β -lactamase II action were proposed by Bickwell & Waley [5] on the basis of cryoenzymology studies. Those authors proposed that a carboxy-group-containing residue, acting as a fifth ligand, functions as a general base to deprotonate the water molecule bound to the zinc ion. On the basis of a group-modification study, Little *et al.* [6] implicated Glu³⁷ as the residue functioning as a general base critical for catalysis. However, Sutton *et al.* [2] found that Glu³⁷ was far removed from the catalytic cluster (His⁸⁶, His⁸⁸, His²¹⁰ and Cys¹⁶⁸) of the cadmium derivative of β -lactamase II at 3.5 nm resolution, and suggested that Glu²¹², which is much closer to the active site, could play the role of a general base proposed by Little *et al.* [6] for Glu³⁷.

Using oligonucleotide mutagenesis to target Glu³⁷ and change it to Gln³⁷, and to convert Glu²¹² into Gln²¹², we have previously shown that neither of these residues is essential for the catalytic activity of β -lactamase II [7]. With the aid of the three-dimensional structure of the active site of *B. cereus* 569/H β -lactamase II [2], we have investigated the possible involvement of other carboxy-group-containing amino acids near the active-site zinc ion to search for residues critical for β -lactamase II function.

MATERIALS AND METHODS

Escherichia coli DH1 (*recA1*, *hsdR17*, *supE44*, *gyrA96*) was used as the host strain for cloning and expression of the β -lactamase II gene. *E. coli* strain CJ236 (*dut*, *ung*, *thi*, *relA*; pCJ105{Cm^r}) was used to generate uracil-containing single-

stranded DNA templates for oligonucleotide-directed mutagenesis [8]. DNA sequencing primers and oligonucleotides for mutagenesis were synthesized with a Biosearch Cyclone DNA synthesizer using the β -cyanoethyl phosphoramidite chemistry described by Caruthers [9]. Oligonucleotides were purified with NENSORB preparative chromatography columns purchased from the du Pont/New England Nuclear Co. Restriction enzymes and bacteriophage T4 DNA ligase were obtained from Bethesda Research Laboratories and used as recommended by the supplier. Sequenase and bacteriophage T4 DNA polymerase were purchased from the United States Biochemical Corp., and [α -³⁵S]thio-dATP from du Pont/New England Nuclear Co.

The measurement of the maximum antibiotic resistance of cells harbouring mutant β -lactamase genes were performed as described previously [7,10]. Schultz & Richards [10] have shown that this method accurately quantifies the level of 'in vivo' resistance achieved by *E. coli* harbouring plasmid pBR322 to several β -lactam antibiotics independently of the inoculum size.

Cloning and vectors

A modified phagemid pTZ19R-tet, derived from pTZ19R[11] was used as single-stranded-DNA-generating vector. The ampicillin-resistance determinant of pTZ19R was insertionally inactivated by subcloning a 1.4 kbp *EcoRI*–*AvqI* DNA fragment containing the tetracycline-resistance gene of pBR322 at the unique *ScaI* site of the vector. The resultant phagemid, designated 'pTZ19R-tet' confers tetracycline, but not ampicillin, resistance to *E. coli* (Fig. 1). The β -lactamase II from *B. cereus* strain 5/B/6 was isolated as a 1.2 kbp *HaeIII* fragment from pUC13-tet(1.2) [7,12] and subcloned at the *SmaI* site of pTZ19R-tet in the orientation that generates the sense strand of the β -lactamase II gene as single-stranded DNA after helper-phage infection (Fig. 1). The resultant phagemid, designated 'pTZ19R-tet(1.2)', confers tetracycline and ampicillin resistance to *E. coli*.

Isolation of single-stranded DNA from pTZ19R-tet(1.2)

Overnight cultures of *E. coli* CJ236 harbouring phagemid pTZ19R-tet(1.2) were diluted 100-fold to inoculate 40 ml of 2XYT liquid medium containing ampicillin (30 μ g/ml), tetracycline (10 μ g/ml) and chloramphenicol (30 μ g/ml). These cultures were grown at 37 °C with aeration to an *A*₆₀₀ of 0.3, infected with M13KO7 helper phage [13] at a multiplicity of infection of 20 phages/bacterium, and incubated for an

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Fig. 1. Phagemid pTZ19R(1.2)

The ampicillin-resistance determinant of phagemid pTZ19R was insertionally inactivated at the unique *ScaI* site by cloning a 1.4 kbp *EcoRI*–*AvaI*-filled-in DNA fragment derived from pBR322 that specifies tetracycline resistance (*tet*). A 1.2 kbp *HaeIII* DNA fragment containing the entire reading frame of *B. cereus* 5/B/6 β -lactamase II was isolated from pUC13-*tet*(1.2) [7,12] and ligated at the unique *SmaI* site within the vector's multiple cloning region. Brackets indicate restriction-nuclease target sequences that were removed as a result of blunt-end ligations in the construction of the chimaeric vector. The approximate locations of the pBR322 origin of replication (*ori*), bacteriophage f1G origin of replication and the alpha-complementing β -galactosidase region (*lacZ*) are indicated by the concentric bold lines. pTZ19R(1.2) expresses tetracycline and ampicillin/cephalosporin C resistance in *E. coli* and generates the sense strand of *B. cereus* 5/B/6 β -lactamase II after infection of the host strain with helper phage M13KO7 [13].

additional 1 h as described above. Kanamycin (50 μ g/ml) was then added to ensure the growth of phage-infected bacteria. Phage-infected cultures were incubated at 37 °C overnight with aeration and centrifuged at 12000 *g* for 10 min at 4 °C. A 10 μ l portion of RNAase A (10 mg/ml; boiled 10 min) was added to the supernatant medium, and the solution was left at room temperature for 30 min. Aliquots of the solution (8 ml each) were transferred to Corex centrifuge tubes. To each tube, 1 ml of 5 M-NaCl and 1 ml of 40 % poly(ethylene glycol) (M_r 8000) were added and the mixtures were left on ice for 1 h. Virions were collected by centrifugation at 12000 *g* for 20 min at 4 °C, each pellet was resuspended in 0.5 ml of TE buffer (10 mM-Tris/HCl/1 mM-EDTA, pH 7.5) and transferred to Microfuge tubes. Single-stranded DNA was isolated from virions by five consecutive phenol and chloroform extractions. After addition of 80 μ l of 7.5 M-ammonium acetate and 1 ml of 95 % (v/v) ethanol, DNA was collected by centrifugation, washed with 70 % ethanol, and dried under vacuum. The DNA pellets were resuspended and pooled in a final volume of 50 μ l of sterile deionized water.

Site-directed mutagenesis and DNA sequencing

Site-directed mutagenesis was performed with the procedure of Kunkel [8]. Synthesis *in vitro* of the plus strand of uracil-containing single-stranded pTZ19R-*tet*(1.2) was performed as described previously [7]. A 10 μ l portion of the '*in-vitro*'-synthesis reaction mixture was used to transform *E. coli* DH1, and tetracycline-resistant colonies were selected. Desired mutants were identified by sequencing supercoiled DNA templates prepared from 1.5 ml overnight cultures of transformants [14,15].

Western-blot analysis of mutant β -lactamase II

Western blotting of β -lactamase II was performed by following published recommendations [16]. Overnight cultures of *E. coli* DH1 harbouring pTZ19R-*tet*(1.2) chimaeras of wild-type and mutant β -lactamase genes were diluted 100-fold in LB liquid medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract and 5 g of NaCl/litre) containing 10 μ g of tetracycline/ml and grown at 37 °C in an orbital shaker to an A_{600} of 1.0. Lysates from 150 μ l of each culture were electrophoresed on a denaturing 12.5 % (w/v) polyacrylamide gel. Portions were transferred to nitrocellulose paper, and β -lactamase II was revealed with an immunoscreening system (Promega Biotech) under conditions recommended by the supplier, using an unadsorbed rabbit polyclonal anti-(β -lactamase II) primary antibody.

RESULTS

The X-ray-crystallographic data published by Sutton *et al.* [2] show that, in addition to Glu²¹², two other carboxy-group-containing amino acid residues lie near the active site of *B. cereus* β -lactamase II: Asp⁸¹ and Asp⁹⁰.

The function of the β -carboxy group of Asp⁸¹ and Asp⁹⁰ was probed by changing both residues to asparagine as described in the Materials and methods section. In the wild-type β -lactamase gene, Asp⁸¹ and Asp⁹⁰ are specified by the codon 5'GAT, which was changed to 5'AAT to substitute asparagine. In addition, the Asp⁹⁰ codon was changed to glutamic acid (5'GAA) to probe the role of the exact spatial position of the β -carboxy group of Asp⁹⁰. These mutations were constructed by synthesis *in vitro* of the plus strand of uracil-containing single-standard DNA template iso-

Fig. 2. DNA sequencing of constructed mutants of β -lactamase II

The codons surrounding point mutations introduced by oligonucleotide-directed point mutations in β -lactamase II were established by DNA sequencing [15]. Residues bearing an asterisk represent the nucleotide substitutions in the mutations. (a) Wild-type (WT) Asp⁹⁰ to Asn⁹⁰; (b) wild-type Asp⁹⁰ to Glu⁹⁰; (c) wild-type Asp⁸¹ to Asn⁸¹.

Fig. 3. Western-blot analysis of mutant β -lactamases

Lysates of *E. coli* DH1 harbouring the cloned Asp⁹⁰ $\rightarrow$ Asn⁹⁰ or Asp⁹⁰ $\rightarrow$ Glu⁹⁰ mutant genes were analysed by Western blotting using an unadsorbed rabbit polyclonal β -lactamase II primary antibody (see the Materials and methods section). Lane 1, control: lysate from *E. coli* DH1[pTZ19R-tet]; non-recombinant vector; lane 2, lysate from *E. coli* DH1[pTZ19R-tet(1.2)]; wild-type *B. cereus* β -lactamase II; lane 3, lysate from *E. coli* DH1[pTZ19R-tet(1.2)]; Asp⁹⁰ $\rightarrow$ Asn⁹⁰ mutant β -lactamase II; lane 4, lysate from *E. coli* DH1[pTZ19R-tet(1.2)]Asp⁹⁰ $\rightarrow$ Glu⁹⁰ mutant β -lactamase II.

lated from *E. coli* CJ236 harbouring the phagemid pTZ19R-tet(1.2) after hybridization of the appropriate mutagenic 23-mer oligonucleotide complementary to the sense strand of the gene. Selection against the wild-type uracil-containing DNA strand in *E. coli* DH1 was so strong that the efficiency of the mutagenesis was more than 50% in all three constructs. The desired mutations were confirmed by DNA sequencing of only three transformants from each construction (Fig. 2).

Phenotype of mutants

The phenotype of each mutant (Asp⁸¹ $\rightarrow$ Asn⁸¹; Asp⁹⁰ $\rightarrow$ Asn⁹⁰; Asp⁹⁰ $\rightarrow$ Glu⁹⁰) was tested by measuring the maximum resistance conferred to *E. coli* DH1 by the mutant β -lactamase II genes on antibiotic concentration gradient plates and compared with the wild-type β -lactamase II gene when harboured in phagemid pTZ19R [7,10]. The results showed that the Asp⁸¹ $\rightarrow$ Asn⁸¹ β -

lactamase retained its capacity to confer ampicillin and cephalosporin C resistance to *E. coli* at levels comparable with the wild-type enzyme 75 μ g/ml and 150 μ g/ml respectively), whereas the Asp⁹⁰ $\rightarrow$ Asn⁹⁰ and Asp⁹⁰ $\rightarrow$ Glu⁹⁰ mutations abolished all detectable ampicillin and cephalosporin C resistance in *E. coli*.

Cleared lysates of *E. coli* DH1 harbouring the cloned Asp⁹⁰ $\rightarrow$ Asn⁹⁰ or Asp⁹⁰ $\rightarrow$ Glu⁹⁰ mutant genes were analysed by Western blotting to verify the presence of unprocessed and mature β -lactamase II. The results are shown in Fig. 3. Both mature (M_r 25000) and unprocessed (M_r 28000) β -lactamase II forms were detected in bacteria expressing the wild-type gene [pTZ19R-tet(1.2); lane 2], in lysates of the Asp⁹⁰ $\rightarrow$ Asn⁹⁰ mutant (lane 3) and in cell lysate of the Asp⁹⁰ $\rightarrow$ Glu⁹⁰ mutant (lane 4). These results indicate that single base changes in these mutants do not significantly affect the synthesis or processing of the expected protein product.

DISCUSSION

The advent of rapid methodologies to create amino acid substitutions in proteins has allowed the development of sound strategies to deduce the significance of a given residue in the active site of an enzyme when crystallographic data are available [17]. One approach assumes that the residues around the active site of the enzyme are likely to be in close contact with the substrate and that their role in catalysis can be discerned by site-specific substitutions generated by oligonucleotide-specific mutagenesis.

We showed previously that neither Glu³⁷ nor Glu²¹², which had been proposed to function as a general base, is essential for the catalytic activity of *B. cereus* 5/B/6 β -lactamase II [7]. Re-examination of the three-dimensional representation of the active site published by Sutton *et al.* [3] revealed no other glutamic acid residue in the vicinity of the catalytic tetrad co-ordinating on the zinc ion. Alternatively, we considered the possibility that the only remaining dicarboxylic acids closest to the active site, namely Asp⁸¹ and Asp⁹⁰, could function as the general base proposed by Bicknell & Waley [5]. To test this hypothesis, Asp⁸¹ and Asp⁹⁰ were changed to asparagine by oligonucleotide-directed mutagenesis and the activity of the mutant genes determined.

Although the Asp⁸¹ $\rightarrow$ Asn⁸¹ mutant β -lactamase II displayed wild-type activity in conferring ampicillin and cephalosporin C

resistance to *E. coli* DH1 on antibiotic gradient plates, the Asp⁹⁰ → Asn⁹⁰ mutant enzyme could not confer any detectable resistance to either antibiotic in *E. coli*. The entire reading frames of both mutant β -lactamase were resequenced using oligonucleotide primers positioning modified T7 DNA polymerase extensions on both template strands to ensure that only the designed substitution could account for the loss of enzyme activity (results not shown). In addition, the protein products of these mutant genes were identified by Western-blot analysis (Fig. 3). These results strongly suggest that the β -carboxy group of Asp⁹⁰ plays an important role in the catalysis of β -lactamase II, which may be that of the general base suspected by Bicknell & Waley, (1985). Consideration of the Asp⁹⁰ → Glu⁹⁰ mutation supports this interpretation. When the secondary carboxy group of Asp⁹⁰ was distanced from the active site by the length of one methylene group, the mutant enzyme could not confer any detectable ampicillin or cephalosporin C resistance to *E. coli*.

It might be argued that the change of Asp⁹⁰ to asparagine disrupts the precise three-dimensional structure of the active site to generate an inactive enzyme. However, asparagine is considered to be a conservative substitution for aspartic acid in terms of size and therefore an improbable effector of catastrophic structural changes. Furthermore, this contention becomes unlikely when we consider the Asp⁸¹ → Asn⁸¹ substitution, which is also very close to the catalytic cluster, but had no discernible effect on enzyme activity. The resolution of this argument will require the elucidation of the three-dimensional structure of the active site in the Asp⁹⁰ → Asn⁹⁰ mutant enzyme and comparison with the wild-type β -lactamase II. A number of Asp → Asn changes do not seem to distort the native structure of proteins. Sprang *et al.* [18], for example, determined the three-dimensional structure of the active site of a mutant trypsin with over 100-fold reduced enzymic activity after changing Asp¹⁰², one of the residues in the active site [19], to asparagine. These authors confirmed that the active site in the mutant trypsin was identical with that in the wild-type.

Additional biochemical and biophysical experiments will be required to establish that Asp⁹⁰ functions as a general base to assist in the hydrolysis of the amide bond of β -lactam substrates.

Examination of kinetic parameters of these mutant β -lactamases should provide other insights to define the exact nature of the reaction mechanism.

This work was supported by a Delaware Research Partnership grant with matching funds provided by Abbott Laboratories, North Chicago, IL, U.S.A., and by a University of Delaware Biomedical Research Support Grant. We thank Dr. Robert W. Shaw for the purified *B. cereus* 569/H β -lactamase, and Dr. Harold White III for critically reading the manuscript.

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