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Expression, purification, crystallization and preliminary X-ray analysis of the native class C β-lactamase from *Enterobacter cloacae* 908R and two mutants

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Crystals have been obtained of the *Enterobacter cloacae* 908R β-lactamase and two point mutants by the vapour-diffusion method using similar conditions [pH 9.0, polyethylene glycol (M_r = 6000) as precipitant]. The three crystal forms belong to the orthorhombic space group $P2_12_12$, with roughly the same unit-cell parameters; *i.e.* for the wild-type crystals a = 46.46, b = 82.96, c = 95.31 Å. In the best cases, the crystals diffract to about 2.1 Å resolution on a rotating-anode X-ray source at room temperature. Co-crystallization experiments of poor substrates with the wild-type protein and the active-site serine mutant (S64C) are planned and should lead to a better understanding of the catalytic mechanism of class C β-lactamases.

1. Introduction

β-Lactamases catalyze the hydrolysis of the lactam bond of β-lactam antibiotics, inactivating these compounds and thus protecting the bacteria that express them. These enzymes have been grouped into four classes (active-site serine β-lactamase classes A, B and C, and Zn^{2+} metallo β-lactamase class B) on the basis of their structures, substrate preferences and inhibition pattern (Bush *et al.*, 1995). In many cases, resistance to β-lactam antibiotics arises from a high level of expression of the plasmid-based TEM class A β-lactamases (penicillinases) or of the chromosomally encoded AmpC class C β-lactamases (cephalosporinases) (Frere, 1995).

The three-dimensional structure of the chromosomal cephalosporinases is well known but their mechanism is still a matter of debate. The first crystal structure reported was that of the *Citrobacter freundii* enzyme in complex with aztreonam (2.5 Å) (Oefner *et al.*, 1990). The authors suggested a catalytic role for a series of amino acids, including Ser64, Lys67, Tyr150 and Lys315. Three-dimensional structures of the *E. cloacae* P99 enzyme (PDB code 2blt) and its complex formed on reaction with a phosphonate monoester inhibitor (PDB entry 1bls) were then determined (Lobkovsky *et al.*, 1993, 1994). More recently, the structure of the AmpC β-lactamase from *Escherichia coli* alone (PDB code 2bls), in complex with the transition-state analogue *m*-aminophenylboronic acid (PDB code 3bls) and with the inhibitor benzo(b)thiophene-2-boronic acid (PDB code 1c3b) were obtained at 2.0, 2.3 and 2.25 Å resolution, respectively (Usher *et al.*, 1998; Powers *et al.*, 1999). Finally, Crichlow *et al.* (1999) have studied the structure of the extended-spectrum class C β-lactamase of *E. cloacae* GC1 at 1.8 Å resolution.

As part of our interest in those enzymes, class C β-lactamase from *E. cloacae* 908R has been expressed, purified and crystallized. Two single mutants (S64C and S84C) of the protein were also produced, purified and crystallized. This work is the first step towards the structural analysis of complexes (transition-state analogues, substrates) with this class C β-lactamase. In addition to their being of interest for mechanistical studies, structures of complexes between class C β-lactamases and synthetic compounds may also provide a rational basis for the design of original antibiotics (Weston *et al.*, 1998; Heinze-Krauss *et al.*, 1998).

2. Material and methods

2.1. Strains, plasmids and mutagenesis

E. cloacae strain Q908R was a gift from Dr W. Zimmermann (Ciba-Geigy, Basel, Switzerland). The strain *E. coli* SNO302, an *ampD*$^-$ derivative of SNO3 (Lindberg & Normark, 1987), was used in the cloning of the *E. cloacae* Q908R *ampC* gene. The plasmids used were derivatives of pBR322 constructed by standard recombinant DNA techniques (Sambrook *et al.*, 1989). The mutagenesis procedure for the substitution of residues Ser84 and active-site Ser64 by cysteine residues was described by Dubus *et al.* (1993).

2.2. Enzyme production and purification

Bacteria were grown for 16 h at 310 K in Terrific Broth medium supplemented with

Table 1
Crystallographic data for the wild-type and two mutant enzymes.

Values in parentheses refer to the highest resolution shell.

Crystal form	Wild-type	S84C	S64C
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)			
a	46.54	82.96	46.07
b	83.45	46.46	82.66
c	95.98	95.31	95.71
Resolution range (Å)	31.49–1.91 (1.98–1.91)	47.66–2.36 (2.51–2.36)	62.56–2.23 (2.30–2.23)
Total No. of observations	81043 (1360)	45145 (2753)	48799 (1211)
No. of unique reflections	24390 (908)	13652 (1196)	14132 (676)
Completeness (%)	81.5 (31.1)	86.8 (46.7)	75.9 (36.5)
$I/\sigma(I)$	7.80 (1.46)	17.48 (2.83)	8.48 (1.24)
R_{sym}† (%)	10.9 (46.4)	5.62 (29.8)	11.0 (52.6)

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h)\rangle$ are the *i*th and mean measurements of the intensity of reflection *h*.

10 mg l^{-1} tetracycline. The cells were pelleted by centrifugation (4000*g*, 20 min), suspended in 10 m*M* sodium phosphate pH 7.0 containing 1%(*v*/*v*) phenylethyl alcohol and 5 m*M* EDTA and submitted to three freeze–thaw cycles to permeabilize the outer membrane. The cells were sedimented at 15 000*g* for 20 min and the supernatant was deposited on a CM-Sepharose column previously equilibrated in 10 m*M* sodium phosphate pH 7.0. The enzyme was eluted with a linear NaCl gradient (0–0.25 *M*) in the same buffer. For both cysteine mutants, 2 m*M* dithiothreitol was added to all buffers except that used for elution from the CM-Sepharose column. After the CM-Sepharose step, the preparations appeared to be at least 95% pure by SDS–PAGE (Jacobs *et al.*, 1992; Dubus *et al.*, 1993; Monnaie *et al.*, 1994).

2.3. Proteins analysis and enzymatic activities

Routinely, protein concentrations were estimated by measuring the absorbance at 280 nm. For pure β-lactamase, a molar absorption of 83 600 M^{-1} cm^{-1} was calculated from the content of tryptophan and tyrosine residues (Cantor & Schimmel, 1980).

β-Lactamase activity was determined by measuring the variation of absorbance upon hydrolysis of the substrate on a Uvikon 860 spectrophotometer interfaced with microcomputers. Substrate hydrolysis was followed at 482 nm for nitrocefin, at 260 nm for other cephalosporins and at 235 nm for penicillins at 310 K.

The β-lactamase activity of the active-site serine S64C mutant totally disappeared upon addition of an excess of 4,4′-dipyridyl disulfide, with a concomitant increase in absorbance at 324 nm, which is characteristic of the reaction of thiols (Grasseti & Murray, 1967).

A detailed kinetic study has been performed by Galleni *et al.* (1988) for the wild-type 908R enzyme. Nitrocefin, cephaloridine, cefazolin, cephalothin and cephalexin are good substrates, with k_{cat} values ranging from 72 to 3000 s^{-1}. Cefuroxime, cefotaxime and cefoxitin exhibit low k_{cat} values (0.01–0.08 s^{-1}) with low K_m values, suggesting a rate-limiting deacylation. Imipenem and aztreonam are even poorer substrates and behave as transient inactivators.

The S84C mutation has no effect on the substrate profile of the enzyme. The active-site S64C mutant exhibits drastically reduced k_{cat}/K_m values on all tested substrates. However, the k_{cat}/K_m decrease did not depend on variations of the same steady-state parameters in all cases. In most cases, the mutation transforms the catalytic properties of the enzyme, replacing a rate-limiting deacylation by a rate-limiting acylation. Thus, one could not visualize the accumulation of acyl-enzyme. The only exception is cefoxitin, for which accumulation of the acyl-enzyme intermediate is still observed (Dubus *et al.*, 1993)

Enzyme stability has been studied. The half-life of the WT enzyme is 18 min at 333 K, whereas the S64C enzyme is slightly more stable (100 min at 333 K). Tests performed with nitrocefin at pH 4.5 and cephaloridine at pH 9.0 showed that the enzymes were stable over the entire pH range.

2.4. Crystallization

Crystallization experiments were carried out at room temperature using the hanging-drop vapour-diffusion method. The concentrations of the purified proteins were adjusted to 10 mg ml^{-1} in 10 m*M* HEPES pH 8.2, 5 m*M* EDTA, 2 m*M* DTT buffer for the S64C and S84C mutants and in 10 m*M* Tris–HCl pH 7.5 buffer for the wild-type protein. Drops were prepared by mixing 3 µl of protein solution with 3 µl of reservoir solution and were allowed to equilibrate against 500 µl of reservoir solution. Crystallization conditions were first investigated using the sparse-matrix sampling kit (Hampton Research) and than fine-tuned. Seeding and cross-seeding techniques were further used to produce large quantities of crystals.

2.5. Data collection

Selected crystals were mounted in thin-walled glass capillaries which were sealed with wax after filling both ends with reservoir solution. X-ray experiments were carried out at 293 K using a Siemens X1000 area detector. The X-ray source was graphite-monochromated Cu $K\alpha$ radiation produced by a Rigaku RU-200 rotating-anode generator operating at 40 kV and 80 mA with a 0.3 mm fine-focus cathode. The detector was at a distance of 130 mm with a 2θ angle of 22°. The data-collection step size was 0.2° between frames with a count time of 70 s per frame. Indexing, integration and scaling of data sets were carried out using the *XENGEN* 2.0 package (Howard *et al.*, 1987). The space group was determined by examining the intensity distribution of the X-ray data.

3. Results and discussion

Using the sparse-matrix sampling kit, crystalline precipitates and irregular small crystals were obtained when polyethylene glycol was used as precipitating agent. Attempts to improve crystal quality were made by using polyethylene glycols of different molecular weights and varying the pH of the reservoir solution. Crystals of both the native enzyme and the S84C mutant were obtained independently. The best results were obtained with 0.1 *M* bicine buffer at pH 9.0 and 30% polyethylene glycol (M_r = 6000) using seeding techniques. Under these conditions, well shaped crystals grew to typical dimensions of 0.3 × 0.3 × 0.15 mm at room temperature within a week. The crystallization of the S64C mutant was initiated under the same conditions by using small crystals of the wild-type protein as micro-seeds. Microcrystals obtained from this cross-seeding were further used to repeat the experiment in order to dilute out the effect of heterogeneous seeds.

These crystals diffracted to 2.1 Å and were stable in the X-ray beam. Crystallographic data of wild-type and both S64C and S84C mutants of class C β-lactamase from *E. cloacae* 908R are summarized in Table 1. The crystals belong to the orthorhombic space group $P2_12_12$. The presence of one monomeric molecule in the asymmetric unit gives a crystal packing parameter (V_M) of 2.35 Å^3 Da^{-1} and a solvent content of 47.7%. These values are within the

frequently observed ranges for protein crystals (Matthews, 1968).

The structure of the S84C mutant has been solved by molecular replacement using the program *AMoRe* (Navaza, 1994) as implemented in the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994). The search model used was the refined 2.3 Å structure of the *Enterobacter cloacae* P99 enzyme (PDB code 1bls) using data in the resolution range 10–2.5 Å. The 361 amino-acid sequence of the molecular probe differs by only five mutations. One clear solution for both the rotation and translation functions was directly obtained with an *R* factor and correlation coefficient of 0.28 and 0.804, respectively. The wild-type and S64C mutant structures were obtained using *AMoRe* (Navaza, 1994) from the starting model of the S84C mutant after introduction of the point mutations. Refinement of all three structures is in progress.

Crystals of the WT and of S84C will be further used to study complexes with inhibitors. Preliminary tests have been performed using transition-state analogues derived from phenylboronic acids. A dramatic change in the rate-limiting step of β-lactam hydrolysis results from the substitution of the active-site serine residue by a cysteine (S64C) (Dubus *et al.*, 1993). Therefore, we intend to use the crystals obtained for this mutant in studies of the mechanism of hydrolysis. A series of slow substrates and inhibitors will be selected on the basis of their kinetic properties (Galleni *et al.*, 1988) to generate the corresponding acyl-enzymes and/or Michaelis complex.

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