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C-terminus mutations of *Acremonium chrysogenum* deacetoxy/deacetylcephalosporin C synthase with improved activity toward penicillin analogs

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Abstract

Deacetoxy/deacetylcephalosporin C synthase (acDAOC/DACS) from *Acremonium chrysogenum* is a bifunctional enzyme that catalyzes both the ring-expansion of penicillin N to deacetoxycephalosporin C (DAOC) and the hydroxylation of the latter to deacetylcephalosporin C (DAC). Three residues N305, R307 and R308 located in close proximity to the C-terminus of acDAOC/DACS were each mutated to leucine. The N305L and R308L mutant acDAOC/DACSs showed significant improvement in their ability to convert penicillin analogs. R308 was identified for the first time as a critical residue for DAOC/DACS activity. Kinetic analyses of purified R308L enzyme indicated its improved catalytic efficiency is due to combined improvements of K_m and k_{cat}. Comparative modeling of acDAOC/DACS supports the involvement of R308 in the formation of substrate-binding pocket.

Keywords: DAOC; DAC; Deacetoxy/deacetylcephalosporin C synthase; C-terminus; Mutagenesis; *Acremonium chrysogenum*; Kinetics

1. Introduction

Cephalosporins are β-lactam antibiotics widely used for the clinical treatment of bacterial infection. The first committed step of cephalosporin biosynthesis in fungi is the ring-expansion of penicillin N to deacetyoxycephalosporin C (DAOC) catalyzed by the bifunctional enzyme deacetoxy/deacetylcephalosporin C synthase [1], which also catalyzes hydroxylation of DAOC to deacetylcephalosporin C (DAC) [1,2]. In prokaryotes, DAOCS and DACS are individual enzymes encoded by separate genes that are apparently homologous [3]. These enzymes are iron(II) and 2-oxoglutarate dependent dioxygenases, related to a family of non-heme oxygenases [4] that include IPNS (isopenicillin N synthase, not 2-oxoglutarate dependent) [5]. Engineering of DAOCS became attractive after the realization that it could lead to improved biosynthetic process for the key intermediate of semi-synthetic cephalosporins, 7-amino-deacetoxycephalosporanic acid (7-ADCA) [6–8]. *Streptomyces clavuligerus* DAOCS (scDAOCS) was first recognized to possess broad substrate specificity (converts substrates other than penicillin N, e.g., penicillin G) [9] and became the major target for research and engineering [6–8,10–12]. However, DAOC/DACS of eukaryotic origin is also interesting in being bifunctional and more adapted in its eukaryotic host.

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Understanding of the catalytic mechanism of DAOCS has been greatly advanced by recent structural and mutational analyses of scDAOCS [13–16]. Residues involved in catalysis and substrates binding have been identified. Those important for prime substrate (penicillin N) binding include R160, R162, R266 and N304 [6,14,17]. The C-terminus of scDAOCS was proposed to modulate the entry and release of substrates, and to co-ordinate catalytic reactions [14,18]. Modifications of various C-terminal residues in the scDAOCS affected both catalytic efficiency and substrate specificity to different degrees [6–8,12]. A recent study of *Acremonium chrysogenum* DAOC/DACS (acDAOC/DACS) [19] also implicated its C-terminal residues (N305 and M306) in substrate selectivity and/or catalytic specificity.

Here we report targeted mutations of N305, R307 and R308 of acDAOC/DACS, and try to compare the relative importance of these residues in controlling substrate specificity and enzyme function. Our results indicate R308 of acDAOC/DACS, not previously identified, is a key residue for the engineering of DAOCS. Comparative modeling of acDAOC/DACS with scDAOCS provides additional support to this conclusion.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich or Fluka. HPLC-grade solvents were purchased from Dikma. G-7-ADCA and 6-APA were provided by Associate Professor Guan-Zhu Xu, Institute of Microbiology, Chinese Academy of Sciences. *Escherichia coli* Ess strain was provided by Professor Arnold L. Demain from Drew University, New Jersey, USA. Primers were synthesized by Runbio, Beijing, PR China. Restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase were purchased from Promega or Takara. Penicillinase was purchased from Becton–Dickinson. QuikChange Site-directed Mutagenesis Kit was purchased from Stratagene.

2.2. Construction of recombinant expression vectors

The acDAOC/DACS encoding gene was previously amplified from *A. chrysogenum* CGMCC 3.3795 and cloned into pGEM-T vector [20]. Its sequence differs from the reported acDAOC/DACS gene by three nucleotides (31G versus T, 388G versus A, 948G versus A) that result in two amino acids alterations (11D versus Y and 130A versus T). Primer pair (5′-GTACCATATGACTTCCAAGGTCCCCGTC-3′ and 5′-TAGGATCCCTAAGTGGCTATAGGAGC-3′) was designed to amplify the WT acDAOC/DACS gene from the T vector template. PCR amplified products (~1 kb) were purified from agarose gels and digested with *Nde*I and *Bam*HI. The digested fragments were ligated into the corresponding sites in pET30a(+) expression vector to give pET-CE. The constructions were confirmed with restriction digests and sequencing.

2.3. Site-directed mutagenesis

QuikChange Site-directed Mutagenesis Kit (Stratagene) was used according to manufacturer's instructions with pET-CE as mutagenesis template. Primers used for mutagenesis were as follows: N305L: 5′-GCGGGAACTATGTCCTCATGCGGAGGGATAAG-3′ and 3′-CGCCCTTGATACAGGAGTACGCCTCCCTATTC-5′; N307L: 5′-GGAACTATGTCAACATGCTCAGGGATAAGCCGGC-3′ and 3′-CCTTGATACAGTTGTACGAGTCCCTATTCGGCCG-5′; N308L: 5′-CTATGTCAACATGCGGCTCGATAAGCCGGCGGC-3′ and 3′-GATACAGTTGTACGCCGAGCTATTCGGCCGCCG-5′. Mutated codons are underlined. They were designed to substitute the codons of N305, R307 and R308 to that of leucine. All mutant constructions were verified by DNA sequencing.

2.4. Expression and purification of WT and mutant DAOC/DACSs

A single colony of *E. coli* BL21 (DE3) transformant containing either WT or mutant DAOC/DACS genes was inoculated into LB (5 ml) supplemented with 50 μg kanamycin ml^{-1}. The cultures were grown at 37 °C and 240 rpm overnight, 1 ml was inoculated in 100 ml of the same media and grown until OD_{600} reached 0.6. Then IPTG was added to give a final concentration of 0.7 mM and the cultures were incubated for another 3 h at 30 °C. Culture flasks were put on ice for 5 min and cells were harvested by centrifugation at 6000*g* for 5 min at 4 °C. Cell pellets were washed and resuspended in 5 ml buffer containing 50 mM Tris–HCl (pH 7.4) and 1 mM dithiothreitol (DTT). Cell suspension was sonicated (JY92-2 D sonicator, Zhenjiang, P.R. China) and the solution was centrifuged at 18,000*g* for 15 min at 4 °C. Supernatants were collected as cell-free extracts, and their protein concentrations were determined by the Bradford assay with bovine serum albumin as reference [21].

The expressed DAOC/DACSs were purified using a procedure similar to that of Lloyd et al. [19]. Cell-free extracts were first loaded onto a DEAE–Cellulose A-52 column (2.5 × 30 cm) equilibrated in buffer A (50 mM Tris–HCl, 1 mM EDTA and 1 mM DTT, pH 8.0) at 1 ml min^{-1}. The column was washed with 200 ml buffer A and eluted with a 100–600 mM NaCl linear gradient. The enzyme fractions were collected and concentrated to 5 ml before loading onto a Sephadex G-75 column (1 × 120 cm) equilibrated in buffer B