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ORIGINAL RESEARCH PAPER

Saturation mutagenesis of *Acremonium chrysogenum* deacetoxy/deacetylcephalosporin C synthase R308 site confirms its role in controlling substrate specificity

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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 and the hydroxylation of the latter to deacetylcephalosporin C. The R308 residue located in close proximity to the *C*-terminus of acDAOC/DACS was mutated to the other 19 amino acids. In the resulting mutant pool, R308L, R308I, R308T and R308V showed significant improvement in their ability to convert penicillin analogs, thus confirming the role of R308 in controlling substrate selectivity, the four amino acids all possess short aliphatic sidechains that may improve hydrophobic interactions with the substrates. The mutant R308I showed the highest reactivity for penicillin G, with 3-fold increase in k_{cat}/K_m ratio and 7-fold increase in relative activity.

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Introduction

The ring-expansion of penicillin N to deacetoxycephalosporin C (DAOC) is catalysed by a bifunctional enzyme deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) (Scheidegger et al. 1984), which also catalyzes hydroxylation of DAOC to deacetylcephalosporin C (DAC) (Samson et al. 1987). Ring-expansion of penicillin G followed by enzymatic removal of its sidechain, or ring-expansion of 6-aminopenicillanic acid (6-APA) is promising bioprocess to produce 7-amino-3-deacetoxycephalosporanic acid (7-ADCA), a key intermediate of semi-synthetic cephalosporins (Velasco et al. 2000). This process demands an engineered DAOCS with altered substrate specificity and increased catalytic activity toward desired substrates. *Streptomyces clavuligerus* DAOCS (scDAOCS) was first recognized to possess broad substrate specificity (converts substrates besides penicillin N, such as penicillin G) (Cho et al. 1998) and became the major target for research and engineering (Chin et al. 2004; Goo et al. 2008a, 2009; Hsu et al. 2004; Wei et al. 2003, 2005). However, DAOC/DACS of eukaryotic origin is also interesting in being bifunctional and more adapted in the industrial eukaryotic host.

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Many mutants of scDAOCS show increased ring-expanding activity towards penicillin G, most mutations were found in a small barrel subdomain (Wei et al. 2003; 2005) or in loops near this subdomain (Hsu et al. 2004), especially in the *C*-terminal residues 304–307 (Chin et al. 2004; Chin and Sim 2002; Goo et al. 2008a, b; Lee et al. 2001; Wei et al. 2003). Studies of *Acremonium chrysogenum* DAOC/DACS (acDAOC/DACS) (Lloyd et al. 1999; Wu et al. 2005) also revealed the importance of its *C*-terminal residues N305, M306 and R308 (the equivalents of residues 304, 305 and 307 in scDAOCS) in substrate selectivity and/or catalytic activity. In our previous research, mutation of acDAOC/DACS R308 (R308L) was found to significantly improve its activity to convert penicillin G (Wu et al. 2005). This was the first time that R308 was identified as a critical residue for acDAOC/DACS activity. To further confirm its role, we prepared a saturation mutagenesis library of R308 of acDAOC/DACS, and evaluated the enzyme activities of the ensuing mutants.

Methods

Site-directed mutagenesis

QuikChange site-directed mutagenesis kit (Stratagene) was used according to manufacturer's instructions with pET-CE (Wu et al. 2005) as mutagenesis template. All mutant constructions were verified by DNA sequencing.

Expression and purification of WT and mutant DAOC/DACS

The mutant enzymes were expressed and purified using similar methods as described by Wu et al. (2005) with minor modifications: the *E. coli* cultures were grown at 25°C and induced with 0.4 mM isopropyl IPTG. The expressed DAOC/DACS were purified using an ÄKTA fast protein liquid chromatography system at 4°C. Cell-free extracts were loaded onto a Resource Q anion-exchange column (6 ml) pre-equilibrated with 30 ml buffer A (50 mM MOPS), 1 mM EDTA and 1 mM dithiothreitol (DTT), pH 8.0). The column was washed with 12 ml buffer A and the expandase was eluted with a 0–200 mM NaCl linear gradient at 0.5 ml min^{-1}. The enzyme fractions were collected and concentrated to 0.5 ml before loading onto a Superdex 75 10/300 GL column (10 × 300 mm) and eluted with 36 ml buffer M (50 mM MOPS and 1 mM DTT, pH 7.4) at 0.5 ml min^{-1}. The active fractions were collected and concentrated into a final volume of 0.5 ml.

Enzyme assays and bioassay of penicillin conversion

The ring-expansion activities of DAOC/DACS on various penicillin analogs were assayed using the same methods as described by Wu et al. (2005). The bioassay of penicillin conversion used a procedure similar to that of Cho et al. (1998).

Spectrophotometric assay of penicillin conversion

A spectrophotometric method similar to that reported previously by Baldwin and Crabbe (1987) was used. The reactions were performed at the same conditions as for bioassays. Relative specific activities for mutated enzymes were calculated as the ratio of absorbance at 260 nm of mutant to that of WT enzyme.

HPLC analyses

A Phenomenex C18 column (3.9 × 300 mm) was used. Separation was performed with conditions similar to those of Chin and Sim (2002). Detection was simultaneously at 220 and 260 nm. The retention times of G-7-ADCA and penicillin G were 22.6 and 23.5 min, respectively.

Kinetic analyses

Kinetic parameters of purified wild type (WT) and mutated enzymes for penicillin G conversion were determined using similar procedures as described for enzyme assay except the reaction time was 30 min. Penicillin G was used from 1 to 10 mM, while the amount of the enzyme was kept constant at 0.7 mg ml^{-1}. The kinetic parameters were calculated via non-linear curve fit by Origin 6.1.

Results

Mutation and expression of WT and mutant acDAOC/DACS

R308 was identified as a critical residue for acDAOC/DACS activity (Wu et al. 2005). The R308L mutant showed 4.3-fold increase in its specific activity to penicillin G. Close examination of the comparative model of acDAOC/DACS and the position of R308 relative to the catalytic center led to the proposal that R308 controls substrate entry and product release (Wu et al. 2005).

The expressed WT and mutant acDAOC/DACS were isolated as cell-free extracts and analyzed by SDS-PAGE (data not shown). All mutant acDAOC/DACS were obtained at high levels as soluble proteins (~30% of total soluble protein) as judged by SDS-PAGE. The enzymes were purified using Resource Q and Superdex 75 column to >90% purity (data not shown). The concentrations of each purified protein were about 2–3 mg ml^{-1} as determined by Bradford assay.

Penicillin analogs conversion measured by bioassay and spectrophotometric assay

The relative specific activities of purified enzymes in the conversion of various penicillin analogs were determined by bioassay (Table 1). The mutants R308L, R308I, R308T and R308V showed significantly enhanced activity to convert penicillin analogs compared to the WT enzyme. Among all tested mutants, R308L and R308I showed the most significant improvement of about 520 and 760%, respectively, for penicillin G conversion. Noticeably, R308L and R308I also showed the broadest substrate specificity and the most improved catalytic activity, being able to convert all penicillin analogs tested. Additionally, all other mutants showed similar percent conversion of 6-APA as the WT enzyme (Table 1b), but no detectable conversion of other penicillin analogs was observed.

Due to the intrinsic differences in diffusion factors and antibiotic activities of different ring expansion products, the bioassay results must be judged with caution (Shibata et al. 1996). Thus a more sensitive

Table 1 Relative specific activities of WT and mutated acDAOC/DACS on (a) penicillin analogs conversion and (b) 6-APA conversion determined by bioassay

(a)

	WT	R308T	R308I	R308V	R308L
Penicillin G	100	346	762	391	516
Carbenicillin	100	NC	194	NC	194
Ampicillin	100	NC	396	NC	311
6-APA	100	126	127	90	100
Penicillin V	100	NC	NC	NC	170
Amoxicillin	NC	NC	NC	NC	100

(b)

	WT	R308A	R308N	R308D	R308C	R308Q	R308E	R308H
6-APA	100	127	75	88	100	75	75	114
	R308K	R308M	R308F	R308P	R308S	R308G	R308W	R308Y
	114	101	127	53	75	105	84	43

The activities for wild type acDAOC/DACS were 55, 52, 60.5, 31.5, 80, 61 nmol product/min.mg protein toward penicillin G, carbenicillin, ampicillin, 6-APA, penicillin V and amoxicillin, respectively (each penicillin analog was provided at 10 mM in assay mixture). The results shown in this table were averages calculated from at least five independent bioassay experiments. *NC* no conversion observed

spectrophotometric method (Baldwin and Crabbe 1987) was used to verify the bioassay results. The spectrophotometric data are listed in Table 2. In the spectrophotometric assay, all mutants and WT acDAOC/DACS exhibited broader conversion spectrum to penicillin analogs, due to higher sensitivity of the method; however, the absolute conversion of ampicillin, amoxicillin, carbenicillin and penicillin V were very low. Overall, the percent conversions determined by spectrophotometric assay showed similar trend as those obtained by bioassay, confirming the significant enhanced activities of R308L, R308I, R308T and R308V relative to the WT enzyme.

Kinetics analysis of mutant acDAOC/DACS

Apparent kinetic parameters of purified WT enzyme, R308L and R308I mutants were determined by HPLC using penicillin G as substrate (Table 3). The mutants showed significantly increased k_{cat} (12-fold for R308I and 8.4-fold for R308L, respectively) and K_m (4.2-fold and 5.5-fold, respectively) relative to those of

Table 2 Relative specific activities of WT and mutant acDAOC/DACS on penicillin analog conversion determined by spectrophotometric assay

	Ampicillin	Amoxicillin	6-APA	Carbenicillin	Penicillin G	Penicillin V
WT	100	100	100	100	100	100
R308L	231	267	130	119	458	222
R308I	276	192	151	124	739	199
R308T	124	170	109	102	374	111
R308V	114	228	101	111	380	122
R308A	111	187	140	95	173	118
R308N	101	111	82	88	151	135
R308D	112	129	123	111	138	177
R308C	112	127	113	113	158	187
R308Q	112	114	122	115	167	160
R308E	61	106	129	108	160	194
R308G	101	116	126	112	113	191
R308H	150	130	110	102	128	135
R308K	78	105	99	101	118	177
R308M	78	127	121	110	140	191
R308F	111	110	99	19	110	184
R308P	106	126	118	110	158	160
R308S	75	168	132	117	103	180
R308W	142	116	128	114	158	177
R308Y	89	118	141	115	122	180

The activities for wild type acDAOC/DACS were 55, 52, 60.5, 31.5, 80, 61 nmol product/min.mg protein toward penicillin G, carbenicillin, ampicillin, 6-APA, penicillin V and amoxicillin, respectively (each penicillin analog was provided at 10 mM in assay mixture). The results shown in this table were averages calculated from at least five independent experiments

Table 3 Kinetic parameters for penicillin G conversion by WT and mutated acDAOC/DACS determined via HPLC

Enzymes	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	Relative% k_{cat}/K_m
Wild-type	0.0057 ± 0.0005	1.9 ± 0.4	3	100
R308I	0.069 ± 0.013	8.2 ± 2.6	8.5	287
R308L	0.048 ± 0.005	11 ± 2	4.5	152

All kinetic parameters shown were the average values ± standard errors determined via non-linear curve fit

WT enzyme. As a result, the k_{cat}/K_m for the ring-expansion of penicillin G by the R308I mutant improved over that of the WT enzyme by about 2.9-fold.

Discussion

7-ADCA is the starting material for many semi-synthetic cephalosporin antibiotics. A direct fermentation process for G-7-ADCA followed by enzymatic removal of its sidechain is a promising strategy to produce 7-ADCA (Velasco et al. 2000). This process demands an engineered DAOCS with altered substrate specificity to convert penicillin G.

Although the structure of acDAOC/DACS is not yet available, but since it shows 57% sequence identity to scDAOCS and the major difference between the two enzymes is that acDAOC/DACS has 20 extra residues at its *C*-terminus, which was shown to have little effect on its enzyme activity (Lloyd et al. 1999), we were able to construct a reliable structural model of acDAOCS/DACS using scDAOCS as template. Most of the key residues for penicillin G binding in scDAOCS catalytic center were conserved in acDAOC/DACS (Goo et al. 2009). Many residues not in the catalytic center were also conserved in both enzymes, this includes the *C*-terminal R308 (R307 in scDAOCS). The crystal structures of scDAOCS in complex with the substrate penicillin G and ampicillin have been determined by two groups (Valegård et al. 2004; Öster et al. 2004). As shown by the crystal structure of the scDAOCS-penicillin G complex (PDB code: 1UOB), R307 was on the surface of the enzyme and was far from penicillin G (above 9.8 Å). So it is obvious that scDAOCS R307 is not located in the catalytic center, but scDAOCS R307L mutant showed slightly increased activity for penicillin G (124%) and significantly increased activity for penicillin V (329%) (Chin et al. 2002). The crystal structure of scDAOCS revealed that R307 resides on the edge of substrate-binding pocket, we speculate that R307 might control the rates of substrate entry and/or product release.

A structural model of acDAOC/DACS with penicillin G was constructed with reasonable confidence by SWISS-MODEL web server (http://swissmodel.expasy.org/) and reported in our previous study (Wu et al. 2005). The comparative model (Fig. 1) indicated that R308 is one of the few residues predicted to point toward the substrate-product path and might play the same role as its counterpart R307 in

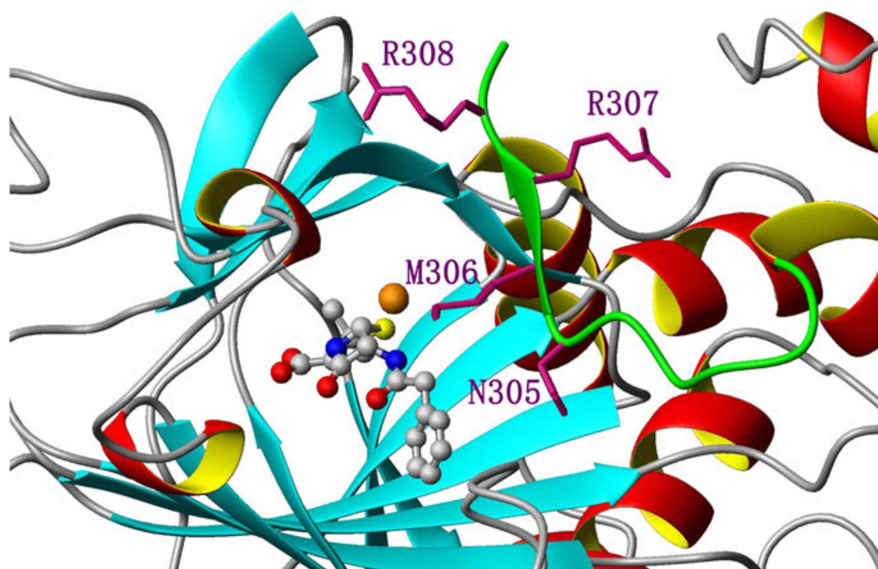

Fig. 1 3D-structure of the modeled substrate binding region of acDAOC/DACS in complex with penicillin G The ferrous ion was displayed as an *orange* ball. Penicillin G was orientated so its tetrahydrothiazole ring points upward. The *C*-terminal residues 300–309 are in *green*. Mutated residue R308 is in *deep pink* and labeled with *purple* numbers. Side-chains of *C*-terminus N305, M306 and R308 point toward the substrate are predicted to influence substrate binding and catalysis; in contrast, R307 extending out of the substrate-binding site should have minimal effect on enzyme activity

scDAOCS in controlling substrate entry and/or product release. This prediction is supported by the activity profiles of the mutants in this study. Among the 19 R308 mutants, R308L, R308V, R308I and R308T showed 3.46- to 7.62-times increased specific activity for penicillin G. The four mutated amino acids (leucine, valine, isoleucine and threonine) have similar short aliphatic sidechains; unlike the positive-charged guanidine containing arginine (Fig. 2). Another R308 mutation to aliphatic alanine (R308A) also increased the activity of mutant to convert 6-APA, penicillin G and amoxicillin as determined by spectrophotometric assay. These results confirm the important role of R308 in substrate pathway control and suggest that the decreased polarity at this position would be beneficial for penicillin G selection.

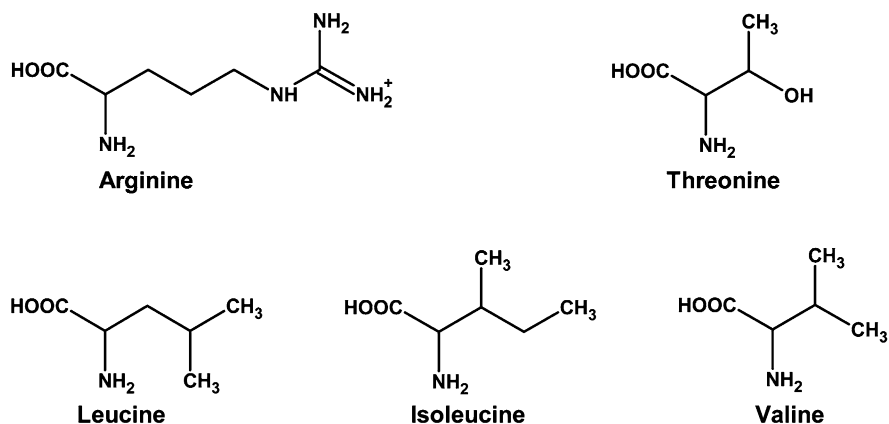

Fig. 2 Structures of the WT and mutated amino acids at position 308

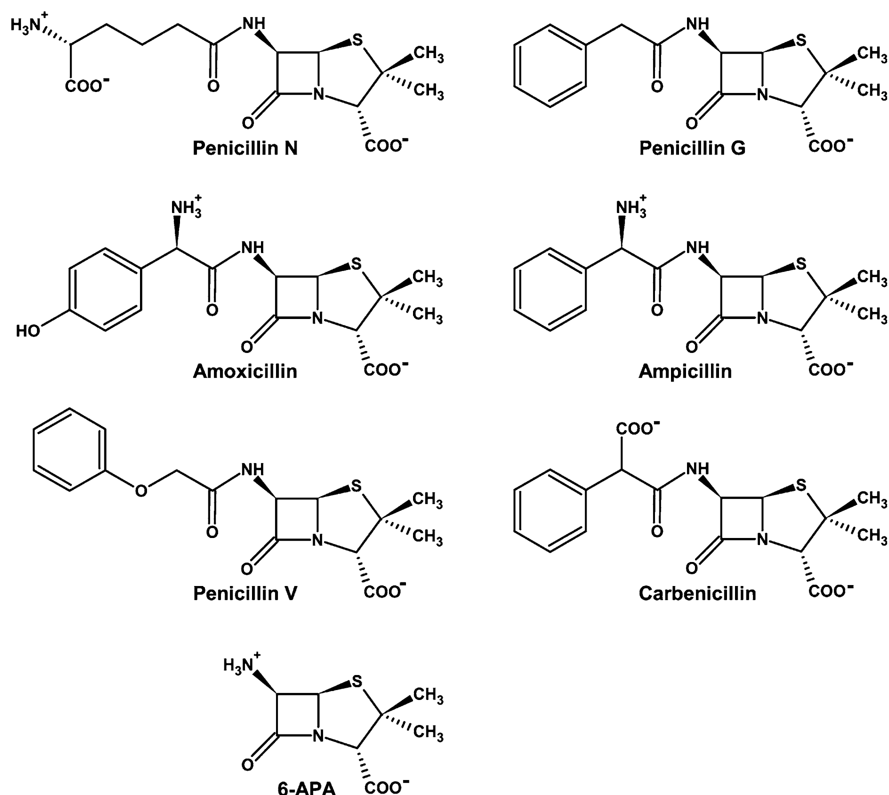

Fig. 3 Chemical structures of penicillin analogs

Mutations to polar or charged residues generated enzymes with activities similar to WT enzyme. Although large aromatic amino acids, such as phenylalanine and tyrosine, may interact well with the side chain of penicillin G, mutations to these amino acids did not generate improved enzymes (Table 2). This maybe due to spacial hindrance caused by their bulky side chains, which could impede substrate entry or product release. Taken together, we conclude that R308 is a critical residue for substrate selection, both side chain polarity and size at this position will influence final enzyme activity.

Among six penicillin analogs tested (Fig. 3), penicillin G has the least sidechain polarity, followed by penicillin V which had a phenoxymethyl sidechain. Other analogs, such as ampicillin, amoxicillin and carbenicillin, have charged amino or carboxyl groups. 6-APA has no sidechain, but an amino group. It is notable that the four hydrophobic mutants of R308 increased the catalytic activity of the enzyme to phenyl-containing penicillin analogs, but had little effect on 6-APA, again suggesting the interaction between the substrate and the enzyme is determined by both polarity and size of the substrate-product path.

The apparent kinetic parameters of WT acDAOC/DACS, R308I and R308L were determined via nonlinear curve fit; both mutants showed significantly increased k_{cat} and K_m (Table 3). These mutations offered appropriate polarity and size for penicillin G selection, and may facilitate substrate entry and/or product release. The large increment in k_{cat} is the direct result of accelerated product release, while K_m increase maybe the result of altered substrate entry rate and secondary conformational changes induced by R308 mutations, since although R308 is far from substrate binding pocket (about 11 Å), it is close to the ferrous ligand H184 (about 5 Å) and two other key residues M181 and M306 in the substrate binding pocket (about 8 and 7 Å, respectively). The overall result is that improved k_{cat} is compromised by simultaneously increased K_m.

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